

FORM PTO-1390  
(REV. 10-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

A-68285/RMS/DHR

U.S. APPLICATION NO. (If known, sect 37 C.F.R. 1.5)

09/762491

INTERNATIONAL APPLICATION NO.

PCT/US99/17776

INTERNATIONAL FILING DATE

06 August 1999 (06.08.99)

PRIORITY DATE CLAIMED

06 August 1998 (06.08.98)

TITLE OF INVENTION

Novel Apoptosis Proteins

APPLICANT(S) FOR DO/EO/US

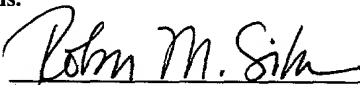
Rigel Pharmaceuticals, Inc.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau. (See enclosed form PCT/IB/308)
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

U.S. APPLICATION NO. (if known, see 37 CFR 1.53) <b>09/762491</b>			INTERNATIONAL APPLICATION NO PCT/US99/17776		ATTORNEY'S DOCKET NUMBER A-68285/RMS/DHR	
17. <input checked="" type="checkbox"/> The following fees are submitted:					CALCULATIONS	PTO USE ONLY
<b>BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1000.00  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$750.00  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00  <div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div>						
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).						
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE			
Total claims	26	-20 =	6	X \$18.00	108.00	
Independent claims	4	-3 =	1	X \$80.00	80.00	
Multiple dependent claims (if applicable)				+ \$270.00	\$270.00	
<b>TOTAL OF ABOVE CALCULATIONS =</b>					1,338.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.					\$669.00	
<b>SUBTOTAL =</b>					669.00	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					\$	
<b>TOTAL NATIONAL FEE =</b>					669.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property					\$	
<b>TOTAL FEES ENCLOSED =</b>					669.00	
					Amount to be:	
					refunded	\$
					charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>669.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>06-1300</u> in the amount of \$ _____ to cover the above fees. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>06-1300 (Order No. A-68285/RMS/DHR)</u> .						
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>						
SEND ALL CORRESPONDENCE TO:  Robin M. Silva, Esq. FLEHR HOHBACH TEST ALBRITTON & HERBERT LLP Four Embarcadero Center, Suite 3400 San Francisco, California 94111-4187 Telephone: (415) 781-1989						
					 SIGNATURE	
					Robin M. Silva NAME	
					38,304 REGISTRATION NUMBER	

09/762491

JC03 Rec'd PCT/PTO

06 FEB 2001

PATENT

Attorney Docket No.: A-68285/RMS/DHR

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Ying Luo, et al.

**NATIONAL PHASE ENTRY OF:**  
PCT/US99/17776

Serial No.: Not Yet Assigned

Filed: Herewith

For: *Novel Apoptosis Proteins*

Assistant Commissioner for Patents  
**BOX PCT**  
Washington, DC 20231

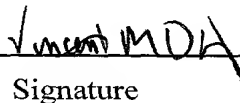
Sir:

**EXPRESS MAIL CERTIFICATION UNDER 37 CFR §1.10**

I hereby certify that this New Application and the documents listed below and referred to as enclosed herein are being deposited with the United States Postal Service on this date February 6, 2001 in an envelope bearing "Express Mail Post Office To Addressee" Mailing Label Number EL 605 085 256 US addressed to: Assistant Commissioner for Patents **Box PCT**, Washington, D.C. 20231.

Vincent Diaz

Person Mailing

  
Signature

The following documents are enclosed herein for filing in the above-identified new application:

1. Transmittal Letter to the U.S. Designated/Elected Office;
2. IB/308;

09/762491-050901

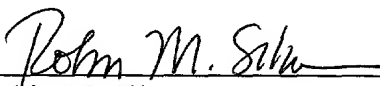
Serial No.: Not Assigned  
Filed: HEREWITH

09/762491  
JC03 Rec'd PCT/PTO 06 FEB 2001

3. Preliminary Amendment
4. Our check in the amount of \$669.00

Respectfully submitted,

FLEHR HOHBACH TEST  
ALBRITTON & HERBERT LLP

  
\_\_\_\_\_  
Robin M. Silva  
Reg. No. 38,304

FLEHR HOHBACH TEST  
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4 Embarcadero Center, Suite 3400  
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Telephone: (415) 781-1989

Dated: 6 February 2001

09/762491

JC03 Rec'd PCT/PTO  
PATENT

06 FEB 2001

Attorney Docket No.: A-68285/RMS/DHR

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Ying Luo, et al.

**NATIONAL PHASE ENTRY OF:**  
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For: *Novel Apoptosis Proteins*

Assistant Commissioner for Patents  
**BOX PCT**  
Washington, DC 20231

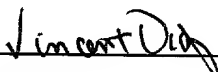
Sir:

**EXPRESS MAIL CERTIFICATION UNDER 37 CFR §1.10**

I hereby certify that the document listed below is being deposited with the United States Postal Service on this date February 6, 2001 in an envelope bearing "Express Mail Post Office To Addressee" Mailing Label Number **EL 605 085 256 US** addressed to: Assistant Commissioner for Patents **Box PCT**, Washington, D.C. 20231.

Vincent Diaz

Person Mailing

  
Signature

**PRELIMINARY AMENDMENT**

Sir:

The Examiner is respectfully requested to enter the following  
amendment:

Serial No.: Not Assigned  
Filed: HERewith

IN THE CLAIMS

Please replace pending claims 1-26 with new replacement claims 1-26.

- 1. A recombinant nucleic acid encoding an Apop3 protein that comprises an amino acid sequence at least 85% identical to the amino acid sequence depicted in Figure 6.
2. A recombinant nucleic acid according to claim 1 comprising the nucleotide sequence depicted in Figure 5 or its complement.
3. A recombinant nucleic acid according to claim 1 wherein said nucleic acid hybridizes under high stringency conditions to the nucleotide sequence depicted in Figure 5 or its complement.
4. A recombinant nucleic acid according to claim 1 wherein said nucleic acid comprises a nucleotide sequence at least 85% identical to the nucleotide sequence depicted in Figure 5 or its complement.
5. A recombinant nucleic acid according to claim 1 wherein said Apop3 protein comprises the amino acid sequence depicted in Figure 6.
6. A recombinant nucleic acid according to claim 1 wherein said Apop3 protein is a human Apop3 protein.
7. A recombinant nucleic acid comprising nucleotides 1-822 depicted in

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Figure 5, or its complement.

8. A recombinant nucleic acid according to claim 1 operably linked to control sequences recognized by a host cell transformed with the nucleic acid.
9. An expression vector comprising the nucleic acid of claim 1.
10. A host cell comprising the nucleic acid of claim 1.
11. A host cell comprising the expression vector of claim 9.
12. A recombinant Apop3 protein comprising an amino acid sequence at least 85% identical to the amino acid sequence depicted in Figure 6.
13. An Apop3 protein according to claim 12 comprising the amino acid sequence depicted in Figure 6.
14. An Apop3 protein according to claim 12 wherein said Apop3 protein is encoded by a nucleic acid comprising the nucleotide sequence depicted in Figure 5 or its complement.
15. An Apop3 protein according to claim 12 wherein said Apop3 protein is encoded by a nucleic acid which nucleic acid comprises a nucleotide sequence at least 85% identical to the nucleotide sequence depicted in Figure 5 or its complement.
16. An Apop3 protein according to claim 12 wherein said Apop3 protein is encoded by a nucleic acid which nucleic acid will hybridize under high stringency conditions to the nucleotide sequence depicted in Figure 5 or its

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**Filed:** HERewith

complement.

17. An Apop3 protein according to claim 12 wherein said Apop3 protein is a human Apop3 protein.
18. A recombinant Apop3 protein comprising the amino acid sequence 1-274 depicted in Figure 6.
19. A process for producing an Apop3 protein according to claim 12 comprising culturing the host cell of claim 10 under conditions suitable for expression of said Apop3 protein.
20. A process according to claim 19, further comprising recovering said Apop3 protein.
21. A monoclonal antibody which specifically binds to an Apop3 protein according to claim 12.
22. A monoclonal antibody according to claim 21 that reduces or eliminates the biological function of said Apop3 protein.
23. A method for screening for a bioactive agent capable of binding to an Apop3 protein according to claim 12, said method comprising:
  - a) combining said Apop3 protein and a candidate bioactive agent; and
  - b) determining the binding of said candidate bioactive agent to said Apop3 protein.
24. A method for screening for a bioactive agent capable of interfering with the binding of an Apop3 protein according to claim 12 and RIP, said method



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comprising:

- a) combining said Apop3 protein, a candidate bioactive agent and a RIP protein; and
- b) determining the binding of said Apop3 protein and said RIP protein.

25. A method for screening for a bioactive agent capable of modulating the activity of an Apop3 protein according to claim 12, said method comprising the steps of:

- a) adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding said Apop3 protein; and
- b) determining the effect of the candidate bioactive agent on apoptosis.

26. A method according to claim 25 wherein a library of candidate bioactive agents is added to a plurality of cells comprising a recombinant nucleic acid encoding said Apop3 protein.--

While no fee is believed to be due, the Commissioner is authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1300 (Our Order No. A-68285/RMS/DHR).

Respectfully submitted,

FLEHR, HOHBACH, TEST,  
ALBRITTON & HERBERT

Dated: 2/6/01

  
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1042392

## NOVEL APOPTOSIS PROTEINS

### FIELD OF THE INVENTION

The invention relates to novel methods and compositions of apoptosis proteins, collectively termed "Apop proteins", and nucleic acids encoding them. The invention further relates to methods of screening for bioactive agents that bind to and modulate Apop protein function for the diagnosis and treatment of disease.

### BACKGROUND OF THE INVENTION

Apoptosis, or programmed cell death, is a highly ordered, genetically controlled process which plays a vital role in both healthy and disease states, including embryogenesis, tissue homeostasis and remodeling, cancer, autoimmune disorders, viral infections, and certain degenerative disorders.

The death domain of TNF receptor-1 (TNFR1) triggers distinct signaling pathways leading to apoptosis and activation of the NF- $\kappa$ B transcription factor through its interaction with the C-terminal death domain of TRADD, a 34 kDa cytoplasmic protein [see Hsu et al., Immunity 4:387-96 (1996)]. TRADD interacts strongly with RIP (receptor-interacting protein; Stanger et al., Cell 81:513-23 (1995), a 74 kDa serine-threonine kinase that with a C-terminal death domain involved in apoptosis; RIP also activates NF- $\kappa$ B. A second RIP protein, RIP2 or RICK [see McCarthy et al., J. Biol. Chem. 273:16968 (1998) and Inohara et al., J. Biol. Chem. 273:12296 (1998)] also contains a death domain and activates NF- $\kappa$ B.

A characteristic feature of apoptosis is activation of a cascade of cytoplasmic proteases that results in the cleavage of selected target proteins. ICE (interleukin 1 beta-converting enzyme) family proteases, also known as caspase proteases, initiate the active phase of apoptosis by degrading specific structural, regulatory, and DNA repair proteins within the target cell [Lazebnik et al., Nature 371:346-7 (1994); Casciola-Rosen et al., J. Biol. Chem. 269:30757-60 (1994)]. For example, a RIP-like kinase, termed CARDIAK/RICK or RIP2 [see Thome et al., Current Biol. 8:885-88 (1998); McCarthy et al., J. Biol. Chem. 273:16968-75 (1998); Inohara et al., J. Biol. Chem. 273:12296-300 (1998)] has been shown to associate with caspase-1. These caspases are related to the *C. elegans* cell death gene product. Caspases are cysteine proteases that display aspartate specificity, and have been shown by a number of researchers to be crucial to apoptotic pathways. For a review, see Cryns et al., Genes & Development 12:1551-70 (1998). The natural substrates of the caspases are key regulatory and

structural proteins, including protein kinases and proteins involved in DNA repair and cytoskeletal integrity.

There are a number of inhibitors of apoptosis (IAPs) that have been identified. Originally identified in baculoviruses, IAPs suppress the host cell death response, thereby allowing survival and propagation of the virus. To date, there are five human IAPs identified, which when expressed in human cells can inhibit apoptosis induced by a variety of stimuli. In addition, the IAPs have been shown to be fairly selective, with different pathways and/or enzymes being inhibited. Human XIAP, cIAP1 and cIAP2 are direct inhibitors of at least two caspase family members, caspase-3 and caspase-7.

Accordingly, the proteins involved in apoptosis and its regulation are of paramount interest, and it is an object of the invention to provide novel apoptosis proteins, herein termed Apop proteins, and in particular Apop1, Apop2, and Apop3 proteins and related molecules. It is a further object of the invention to provide recombinant nucleic acids encoding Apop proteins, and expression vectors and host cells containing the nucleic acid encoding them. A further object of the invention is to provide methods for screening for antagonists and agonists of Apop proteins.

#### SUMMARY OF THE INVENTION

According to the objects outlined above, the present invention provides recombinant nucleic acids encoding Apop proteins, and in particular Apop1, Apop2 and Apop3 proteins, that are at least about 85% identical to the amino acid sequence depicted in Figure 2, Figure 4, and Figure 6, respectively. Similarly provided are recombinant nucleic acids at least about 85% identical to the nucleic acid sequence depicted in Figures 1, 3, and 5 or their complements. Expression vectors and host cells comprising the nucleic acids are also included.

In a further aspect, the invention provides methods of making Apop proteins, comprising providing a cell comprising an Apop protein encoding nucleic acid and subjecting the cell to conditions which allow the expression of Apop proteins.

In an additional aspect, the invention provides recombinant Apop proteins, that are at least about 85% identical to the amino acid sequences depicted in Figures 2, 4, and 6, respectively, and antibodies that will bind to the Apop1 proteins, Apop2 proteins and Apop3 proteins.

In a further aspect, the invention provides monoclonal and polyclonal antibodies binding to the apoptosis proteins Apop1, Apop2, and Apop3.

In a further aspect, the present invention provides methods for screening for a bioactive agent capable of binding to an Apop protein. The method comprises combining a candidate bioactive agent and an Apop protein and determining the binding of the candidate agent to the Apop protein.

5 In an additional aspect, the present invention provides methods for screening for agents capable of interfering with the binding of Apop3 and RIP. The methods comprise combining an Apop3 protein, a candidate bioactive agent and a RIP protein, and determining the binding of the Apop3 protein and the RIP protein.

10 In an additional aspect, the present invention provides methods for screening for agents capable of interfering with the binding of Apop1 and XIAP or Apop2 and XIAP. The methods comprise combining an Apop1 protein or Apop2 protein, a candidate bioactive agent and an XIAP protein, and determining the binding of the Apop1 protein and the XIAP protein or Apop2 protein and the XIAP protein.

15 In an additional aspect, the invention provides methods for screening for an bioactive agent capable of modulating the activity of an Apop protein. The method comprises the steps of adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding an Apop protein and determining the effect of the candidate bioactive agent on apoptosis.

20 In one aspect, a method for screening for a bioactive agent comprises providing a cell that expresses an expression profile gene selected from the group consisting of the expression profile genes set forth in Figures 1, 3, and 5.

25 In another aspect, a method for screening for a bioactive agent, comprises providing a cell that expresses an expression profile protein selected from the group consisting of the expression profile proteins having the amino acid sequence set forth in Figures 2, 4, and 6.

In another aspect, the invention provides a biochip comprising recombinant nucleic acids encoding Apop proteins. The recombinant nucleic acids, encoding Apop proteins and bound to the biochip, may be at least about 85% identical to the nucleic acid sequence depicted in Figures 1, 3, and 5 or their complements.

Other aspects of the invention will become apparent to the skilled artisan by the following description of the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleotide sequence of human Apop1. The putative translation start codon (ATG) and translation termination codon (TGA) are underlined.

Figure 2 depicts the amino acid sequence of human Apop1.

Figure 3 depicts the nucleotide sequence of human Apop2. The putative translation start codon (ATG) and the translation termination codon (TGA) are underlined.

Figure 4 depicts the amino acid sequence of human Apop2.

Figure 5 depicts the nucleotide sequence of human Apop3.

Figure 6 depicts the amino acid sequence of human Apop3.

Figure 7 depicts a schematic representation of Apop3 truncation mutants. The shaded area depicts the kinase homology domain. Apop3(K50D) has a K to D mutation at amino acid 50. WT, wild-type Apop3, i.e., the full-length protein.

Figure 8 depicts a summary of binding results of Apop3 to RIP in the yeast two hybrid system. +++, very strong interaction; ++, strong interaction; +, detectable interaction; -, no detectable interaction.

Figure 9 depicts the activation of apoptosis by Apop3 in Phoenix-A cells. Empty vector (Vector), Apop3, or Apop3 (1-436) (3 µg) was co-transfected with pGDB (1 µg) into Phoenix-A cells. Hoechst stained apoptotic Phoenix-A cells were examined and counted by fluorescence microscopy. The data are expressed as percentage of apoptotic cells among the total number of cells counted.

Figure 10 depicts the activation of NFκB by Apop3 in Phoenix-A cells. NFκB reporter activity was performed by transiently co-transfecting Phoenix-A cells with the indicated Apop3 expression vectors (3 µg), NFκB-dependent luciferase reporter plasmid (1 µg), and Renilla Luciferase expression vector (0.13 µg). Calcium phosphate precipitation method was used for transfection. The level of expressed tagged proteins was also monitored by Western blot analysis.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel apoptosis proteins and nucleic acids which were initially identified using a yeast two-hybrid screening method. Traditionally, protein-protein interactions were

evaluated using biochemical techniques, including chemical cross-linking, co-immunoprecipitation and co-fractionation and -purification. Recently genetic systems, including the "yeast two-hybrid system" have been described to detect protein-protein interactions. The basic system requires a protein-protein interaction between a "bait protein" and a "test protein" in order to turn on transcription of a reporter gene. See Fields et al., *Nature* 340:245 (1989); Vasavada et al., *Proc. Natl. Acad. Sci. U.S.A.* 88:10686 (1991); Fearon et al., *Proc. Natl. Acad. Sci. U.S.A.* 89:7958 (1992); Dang et al., *Mol. Cell. Biol.* 11:954 (1991); Chien et al., *Proc. Natl. Acad. Sci. U.S.A.*, 88:9578 (1991); U.S. Patent Nos. 5,283,173, 5,667,973, 5,468,614, 5,525,490, and 5,637,463; and U.S. patent application S.N. 09/050,863. In particular Apop1 and Apop2 were identified using XIAP as the "bait protein". Apop3 was identified using RIP as the "bait protein".

Thus, the present invention provides novel apoptosis proteins, termed Apop proteins and nucleic acids encoding them. Unless otherwise explicitly stated herein, the terms "Apop", "Apop protein" or grammatical equivalents thereof include Apop1 proteins, Apop2 proteins, and Apop3 proteins, the wild-type amino acid sequences of which are depicted in Figure 2, Figure 4, and Figure 6, respectively. Similarly, the terms "Apop nucleic acid", "Apop DNA", "Apop nucleotide sequence" or grammatical equivalents thereof include nucleic acids which encode Apop1 proteins, Apop2 proteins and Apop3 proteins, the wild-type nucleic acid sequences of which are depicted in Figure 1, Figure 3, and Figure 5, respectively.

In a preferred embodiment, the Apop proteins are from vertebrates and more preferably from mammals, including rodents (rats, mice, hamsters, guinea pigs, etc.), primates, farm animals (including sheep, goats, pigs, cows, horses, etc) and in the most preferred embodiment, from humans. However, using the techniques outlined below, Apop proteins from other organisms may also be obtained.

An Apop protein of the present invention may be identified in several ways. "Protein" in this sense includes proteins, polypeptides, and peptides. An Apop nucleic acid or Apop protein is initially identified by substantial nucleic acid and/or amino acid sequence homology to the sequences shown in Figures 1, 2, 3, 4, 5, and 6. Such homology can be based upon the overall nucleic acid or amino acid sequence.

As used herein, a protein is an "Apop protein" if the overall homology of the protein sequence to the amino acid sequences shown in Figures 2, 4, and 6 is preferably greater than about 75%, more preferably greater than about 80%, even more preferably greater than about 85% and most preferably greater than 90%. In some embodiments the homology will be as high as about 93 to 95 or 98%.

Homology in this context means sequence similarity or identity, with identity being preferred. This homology will be determined using standard techniques known in the art, including, but not limited to, the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc Natl. Acad. Sci. U.S.A.* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), or the Best Fit sequence program described by Devereux et al., *Nucl. Acid Res.* 12:387-95 (1984), preferably using the default settings, or by inspection.

In a preferred embodiment, similarity is calculated by FastDB based upon the following parameters: mismatch penalty of 1.0; gap size penalty of 0.33, joining penalty of 30.0 ("Current methods in Comparison and Analysis", *Macromolecule Sequencing and Synthesis*, selected methods and Applications, pp. 127-149 (1998), Alan R. Liss, Inc.). Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle, *J. Mol. Evol.* 35:351-60 (1987); the method is similar to that described by Higgins and Sharp *CABIOS* 5:151-3 (1989). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

An additional example of a useful algorithm is the BLAST algorithm, described in Altschul et al., *J. Mol. Biol.* 215: 403-410 (1990) and Karlin et al., *Proc. Natl. Acad. Sci. U.S.A.* 90:5873-87 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al., *Methods in Enzymology* 266:460-480 (1996); [http://blast.wustl.edu/blast/ README.html](http://blast.wustl.edu/blast/README.html)]. WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity. A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

In a similar manner, "percent (%) nucleic acid sequence identity" with respect to the coding sequence of the polypeptides identified herein is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the coding sequence of the Apop proteins (see Figures 1, 3, and 5). A preferred method utilizes the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

An additional useful algorithm is gapped BLAST as reported by Altschul et al., Nucl. Acid Res. 25:3389-3402 (1997). Gapped BLAST uses BLOSUM-62 substitution scores; threshold  $T$  parameter set to 9; the two-hit method to trigger ungapped extensions; charges gap lengths of  $k$  a cost of  $10+k$ ;  $X_u$  set to 16, and  $X_g$  set to 40 for database search stage and to 67 for the output stage of the algorithms. Gapped alignments are triggered by a score corresponding to ~22 bits.

The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the protein sequences shown in Figures 2, 4, and 6 it is understood that the percentage of homology will be determined based on the number of homologous amino acids in relation to the total number of amino acids. Thus, for example, homology of sequences shorter than that shown in Figures 2, 4, and 6, as discussed below, will be determined using the number of amino acids in the shorter sequence.

In a preferred embodiment, the Apop protein binds to an IAP protein. Inhibitor-of-apoptosis (IAP) proteins are a novel family of anti-apoptotic proteins that were first identified in baculoviruses. Members of the human IAP family include, e.g., NAIP, HIAP-1 (human inhibitor of apoptosis protein-1), HIAP-2 (human inhibitor of apoptosis protein-2), c-IAP-1, c-IAP-2, and XIAP. They are thought to inhibit cell death via direct inhibition of caspases. Indeed, some IAP family proteins, e.g., c-IAP-1, c-IAP-2 and XIAP can bind to and inhibit the distal cell death proteases, caspases-3 and -7 (Roy et al., EMBO J., 16:6914-25 (1997); Takahashi et al., J. Biol. Chem. 273:7787-90 (1998)). Although IAPs are highly conserved through evolution, the mechanisms by which they interfere with the apoptotic cell death are not clear. There may be a number of unknown protein substrates for IAPs and agonistic or antagonistic proteins modulating the activity of IAPs.

In order to identify novel proteins interacting with IAPs, in particular with XIAP, we employed the yeast two-hybrid screening system. Using XIAP as the "bait protein" we identified two novel proteins interacting with XIAP, named Apop1 and Apop2.

In a preferred embodiment, the Apop protein is an Apop1 protein. The nucleotide sequence of Apop1 is depicted in Figure 1 and the encoded protein in Figure 2. A significant portion of the Apop1 nucleic



acid and the encoded Apop1 protein has identity to human cathepsin B precursor protein and the corresponding DNA [Ritonja et al., FEBS Lett. 181:169-172 (1985); Chan et al., Proc Natl. Acad. Sci. U.S.A. 83:7721-7725 (1986); Fong et al., Proc Natl. Acad. Sci. U.S.A. 83:2909-2913 (1986); Moin et al., Biochem. J. 285:427-434 (1992); Cao et al., Gene 139:163-169 (1994)]. Cathepsin B is a lysosomal thiol proteinase that may have additional extralysosomal functions. In particular, nucleotides 364 to 716 of the Apop1 nucleotide sequence depicted in Figure 1 have >99% (352/353 nucleotide residues) identity to human cathepsin B (CTSB) mRNA (GenBank accession numbers NM\_001908 and M14221). Likewise, on the amino acid level, the Apop1 amino acid sequence depicted in Figure 2, shows 100% (109/109 amino acid residues) identity to human cathepsin B precursor protein (GenBank accession number P07858) and >99% (108/109 amino acid residues) identity to human cathepsin B precursor protein (GenBank accession number NP\_001899). However, the remainder of the Apop1 protein and nucleic acid do not exhibit any homology to the known sequences. Thus the Apop1 nucleic acid sequence of the present invention may represent an alternatively spliced cathepsin B mRNA transcript and the encoded protein may have other biological activities when compared to cathepsin B.

On the nucleic acid level, there are a number of reported sequences including EST99543 and EST41374 that have homology to Apop1.

In a preferred embodiment, the Apop protein is an Apop2 protein. The nucleotide sequence of Apop2 is depicted in Figure 3 and the encoded protein in Figure 4. Apop2 nucleic acid and the encoded Apop2 protein have identity to human cathepsin F precursor protein and the corresponding DNA [Wang et al., J. Biol. Chem. 273:32000-8 (1998); Nagler et al., Biochem. Biophys. Res. Commun. 257:313-8 (1999); Santamaria et al., J. Biol. Chem. 274:13800-9 (1999); Wex et al., Biochem. Biophys. Res. Commun. 259:401-7 (1999); GenBank accession numbers AF071748, NM\_003793, AF088886, AJ007331, and AF132894]. Cathepsin F is a novel papain-like cysteine proteinase, synthesized as a precursor protein, including a hydrophobic signal sequence, a pro-domain, and a catalytic region [Santamaria et al., J. Biol. Chem. 274:13800-9 (1999)]. Surprisingly, Apop2 has strong homology to several other cathepsins. Protein sequence comparison revealed 58% homology with cathepsin W; about 42-43% with cathepsin L, K, S, H, and O; and 38% with cathepsin B [Wang et al., J. Biol. Chem. 273:32000-8 (1998)]. The identification of Apop2 (cathepsin F) by binding to XIAP, represents the first time that a protein containing cathepsin homology has been shown to play a role in apoptosis.

On the nucleic acid level, there are a number of reported EST sequences that comprise portions of the Apop2 nucleic acid. These include EST43911/AA38898, yp01g08.r1/H39591, and EST91038/

AA378321. In addition, there are several areas of homology to known cDNAs, including: 88% identity to a mouse mammary gland cDNA; (AA475592), 95% identity to a human cDNA clone (H15748), and a 88% identity to a mouse mammary gland cDNA (AA958896).

In a preferred embodiment, the Apop protein is an Apop3 protein. Using RIP as the "bait protein" in a yeast two-hybrid screening, Apop3 was identified. The nucleotide sequence of Apop3 is depicted in Figure 5 and the encoded protein in Figure 6. The human Apop3 appears to be expressed in heart, liver, pancreas, placenta and lung, but either weakly or not at all in brain. Apop3 has also been termed "RIP3" [see Yu et al., Current Biology 9:539-42 (1999); Sun et al., J. Biol. Chem. 274:16871-5 (1999)]. The N-terminal portion of Apop3, in particular amino acid residues 1-274, has homology to the kinase domain of RIP (34% identity and 60% similarity) and RIP2 (31% identity and 58% similarity) [see Sun et al., J. Biol. Chem. 274:16871-5 (1999)]. However, the C-terminal portion of Apop3 has no significant homology to any known proteins. Accordingly, Apop3 proteins may be identified in one aspect by significant homology to areas other than the kinase domain. This homology is preferably greater than about 60%, with greater than about 70 or 75% being particularly preferred and greater than about 80% being especially preferred. In some cases the homology will be greater than about 90 to 95 or 98%.

In addition, an Apop3 protein preferably also has significant homology to the kinase domain as described herein. This homology is preferably greater than about 75%, with greater than about 80% being particularly preferred and greater than about 85% being especially preferred. In some cases the homology will be greater than about 90 to 95 or 98%.

Apop proteins of the present invention may be shorter or longer than the amino acid sequences shown in Figures 2, 4, and 6. Thus, in a preferred embodiment, included within the definition of Apop proteins are portions or fragments of the sequences depicted herein. Portions or fragments of Apop proteins are considered Apop proteins if a) they share at least one antigenic epitope; or b) have at least the indicated homology; or c) preferably have Apop biological activity, e.g., including, but not limited to kinase activity, cell death activity, binding to XIAP or RIP, etc.

Well-known methods, such as in vitro manipulations of nucleic acids or PCR (polymerase chain reaction) are known in the art and are routinely used to generate portions or fragments of a desired nucleic acid sequence which then encodes a portion or a fragment of the desired protein. Generally, such portions of an Apop protein comprise at least 10%, preferably at least 20%, more preferably at least 25%, even more preferably at least 30% and most preferably at least 50% of the Apop protein.

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In some embodiments, a portion or fragment of an Apop protein will comprise as much as 60 to 90 or 95% of the respective Apop protein.

In one embodiment, the portion or fragment of the respective Apop protein is contiguous to the Apop protein from which it is obtained. In another embodiment, the portion or fragment of the respective Apop protein is not contiguous to the Apop protein from which it is derived. In this embodiment, two or more parts of the Apop protein, for example, a fragment obtained from the N-terminus and a fragment obtained from the C-terminus are linked, whereby internal sequences are deleted.

In a preferred embodiment, the Apop proteins are derivative or variant Apop proteins. That is, as outlined more fully below, the derivative Apop peptide will contain at least one amino acid substitution, deletion or insertion, with amino acid substitutions being particularly preferred. The amino acid substitution, insertion or deletion may occur at any residue within the Apop peptide.

In addition, as is more fully outlined below, Apop proteins can be made that are longer than those depicted in Figures 2, 4, and 6, for example, by the addition of epitope or purification tags, the addition of other fusion sequences, etc.

Apop proteins may also be identified as being encoded by Apop nucleic acids. Thus, Apop proteins are encoded by nucleic acids that will hybridize to the sequence depicted in Figures 1, 3, and 5 or its complement, as outlined herein.

In one preferred embodiment, the Apop proteins of the present invention may be used to generate polyclonal and monoclonal antibodies to Apop proteins, which are useful as described herein. The terms "Apop antibodies", "antibodies binding to Apop" or grammatical equivalents thereof include antibodies binding to Apop1 proteins, Apop2 proteins, and Apop3 proteins.

Apop antibodies usually are generated with an Apop protein having the amino acid sequence depicted in Figures 2, 4, and 6. In a preferred embodiment, Apop proteins corresponding to a portion or fragment of an Apop protein of which the amino acid sequence is depicted in Figures 2, 4, and 6, are used to generate antibodies. Methods for the preparation and purification of monoclonal and polyclonal antibodies are known in the art and e.g., are described in Harlow and Lane, *Antibodies: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1988). When the Apop protein is used to generate antibodies, the Apop protein must share at least one epitope or determinant with the full length protein shown in Figures 2, 4, and 6. By "epitope" or "determinant" herein is meant a

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portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to a smaller Apop3 protein will be able to bind to the full length protein.

In a preferred embodiment, the epitope is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity to other proteins. The term "antibody" includes antibody fragments, as are known in the art, including Fab Fab<sub>2</sub>, single chain antibodies (Fv for example), chimeric antibodies, etc., either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA technologies. The term "antibody" further comprises polyclonal antibodies and monoclonal antibodies, which can be agonist or antagonist antibodies.

The Apop antibodies of the invention specifically bind to Apop proteins. By "specifically bind" herein is meant that the antibodies bind to the protein with a binding constant in the range of at least  $10^{-4}$ -  $10^{-6}$  M<sup>-1</sup>, with a preferred range being  $10^{-7}$  -  $10^{-9}$  M<sup>-1</sup>.

In a preferred embodiment, an Apop protein of the present invention may be identified by its immunological activity, i.e., its ability to bind to an antibody specific for an epitope found within a protein comprising the amino acid sequence depicted in Figures, 2, 4, and 6. The term "immunological activity" means the ability of the protein to cross react with an antibody which is specific for the protein comprising the amino acid sequence depicted in Figures 2, 4, and 6, i.e., an Apop protein antibody. Accordingly, a protein is an Apop protein, if the protein displays the immunological activity of a protein comprising the amino acid sequence depicted in Figures 2, 4, and 6.

In a preferred embodiment, Apop antibodies are provided. The antibodies may be polyclonal or monoclonal. In a preferred embodiment, the antibodies to Apop are capable of reducing or eliminating the biological function of Apop, as is described below. That is, the addition of anti-Apop antibodies (either polyclonal or preferably monoclonal) to Apop (or cells containing Apop) may reduce or eliminate the Apop activity. Generally, at least a 25% decrease in activity is preferred, with at least about 50% being particularly preferred and about a 95-100% decrease being especially preferred.

Monoclonal antibodies are directed against a single antigenic site or a single determinant on an antigen. Thus monoclonal antibodies, in contrast to polyclonal antibodies, which are directed against multiple different epitopes, are very specific. Monoclonal antibodies are usually obtained from the supernatant of hybridoma culture (see Kohler and Milstein, *Nature* 256:495-7 (1975); Harlow and Lane, *Antibodies: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1988).

In a preferred embodiment, the antibodies to Apop proteins are humanized. Using current monoclonal antibody technology one can produce a humanized antibody to virtually any target antigen that can be identified [Stein, Trends Biotechnol. 15:88-90 (1997)]. Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., *supra*; Riechmann et al., *supra*; and Verhoeven et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Additional examples of humanized murine monoclonal antibodies are also known in the art, e.g., antibodies binding human protein C [O'Connor et al., Protein Eng. 11:321-8 (1998)], interleukin 2 receptor [Queen et al., Proc. Natl. Acad. Sci., U.S.A. 86:10029-33 (1989)], and human epidermal growth factor receptor 2 [Carter et al., Proc. Natl. Acad. Sci. U.S.A. 89:4285-9 (1992)]. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol. 227:381 (1991); Marks et al., J. Mol. Biol. 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol. 147(1):86-95 (1991)). Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10:779-783 (1992); Lonberg et al. *Nature* 368:856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14:845-51 (1996); Neuberger, *Nature Biotechnology* 14:826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995).

In a preferred embodiment, Apop nucleic acids are provided. An Apop nucleic acid of the present invention may be identified by its sequence identity to the nucleotide sequence depicted in Figures 1, 3, or 5 and may be referred to as having some "percent (%)" sequence identity to all or a portion of the nucleotide sequence depicted in Figures 1, 3, or 5. Sequence identity, when referring to nucleic acid, means that the sequences being compared have nucleotides at corresponding positions which are identical. The sequence identity of an Apop nucleic acid is commensurate with the sequence identity of Apop proteins but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the sequence identity for Apop nucleic acids may differ from the sequence identity for Apop proteins. Thus, the nucleic acid sequence homology may be either lower or higher than that of the protein sequence. Thus the homology of the nucleic acid sequence as compared to the nucleic acid sequences of Figures 1, 3, and 5 is preferably greater than 75%, more preferably greater than about 80%, particularly greater than about 85% and most preferably greater than 90%. In some embodiments the homology will be as high as about 93 to 95 or 98%.

In a preferred embodiment, an Apop nucleic acid encodes an Apop protein. As will be appreciated by those in the art, due to the degeneracy of the genetic code, an extremely large number of nucleic acids may be made, all of which encode the Apop proteins of the present invention. Thus, having identified a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids, by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the Apop.

In one embodiment, the nucleic acid homology is determined through hybridization studies. Thus, for example, nucleic acids which hybridize under high stringency to the nucleic acid sequences shown in Figures 1, 3, and 5 or their complements are considered an Apop gene. High stringency conditions are known in the art; see for example Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989) and Ausubel et al., *Short Protocols in Molecular Biology* (John Wiley & Sons, Inc., 1995), both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.

In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Sambrook et al., *supra*, Ausubel et al., *supra*, and Tijssen, *supra*.

The Apop proteins and nucleic acids of the present invention are preferably recombinant. As used herein, and defined below, "nucleic acid" may refer to either DNA or RNA, or molecules which contain both deoxy- and ribonucleotides. The nucleic acids include genomic DNA, cDNA and oligonucleotides including sense and anti-sense nucleic acids. Such nucleic acids may also contain modifications in the ribose-phosphate backbone to increase stability and half life of such molecules in physiological environments.

The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand ("Watson") also defines the sequence of the other strand ("Crick"); thus the sequences depicted in Figures 1, 3, and 5 also include the complement of these sequences. By the term "recombinant

nucleic acid" herein is meant nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid by endonucleases, in a form not normally found in nature. Thus an isolated Apop nucleic acid, in a linear form, or an expression vector formed *in vitro* by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e. using the *in vivo* cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of an Apop protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of a inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Alternatively, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid substitutions, insertions and deletions, as discussed below.

Also included within the definition of Apop proteins of the present invention are amino acid sequence variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the Apop protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the recombinant DNA in cell culture as outlined above. However, variant Apop protein fragments having up to about 100-150 residues may be prepared by *in vitro* synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the Apop protein amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue,



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although variants can also be selected which have modified characteristics as will be more fully outlined below.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed Apop variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants is done using assays of Apop protein activities.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the Apop protein are desired, substitutions are generally made in accordance with the following chart:

Chart I

<u>Original Residue</u>	<u>Exemplary Substitutions</u>
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the Apop proteins as needed. Alternatively, the variant may be designed such that the biological activity of the Apop protein is altered. For example, glycosylation sites may be altered or removed. Similarly, e.g., mutations within the cysteine protease domain of Apop1 or Apop2 or within the kinase domain of Apop3 may be made.

Covalent modifications of Apop polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of an Apop polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N-or C-terminal residues of an Apop polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking Apop to a water-insoluble support matrix or surface for use in the method for purifying anti-Apop antibodies or screening assays, as is more fully described below. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimide.

Other modifications include deamidation of glutamyl and asparaginy residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman &

Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the Apop polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apop polypeptide, and/or adding one or more glycosylation sites that are not present in the native sequence Apop polypeptide.

Addition of glycosylation sites to Apop polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apop polypeptide (for O-linked glycosylation sites). The Apop amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apop polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the Apop polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apop polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of Apop comprises linking the Apop polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Apop polypeptides of the present invention may also be modified in a way to form chimeric molecules comprising an Apop polypeptide fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, such a chimeric molecule comprises a fusion of an Apop polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the Apop polypeptide. The presence of such epitope-tagged forms of an Apop polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apop polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In an alternative embodiment, the chimeric molecule may comprise a fusion of an Apop polypeptide with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)].

Also included with the definition of Apop protein are other Apop proteins of the Apop family, and Apop proteins from other organisms, which are cloned and expressed as outlined below. Thus, probe or degenerate polymerase chain reaction (PCR) primer sequences may be used to find other related Apop proteins from humans or other organisms. As will be appreciated by those in the art, particularly useful probe and/or PCR primer sequences include the unique areas of the Apop nucleic acid sequence. As is generally known in the art, preferred PCR primers are from about 15 to about 35 nucleotides in length, with from about 20 to about 30 being preferred, and may contain inosine as needed. The conditions for the PCR reaction are well known in the art.

In another preferred embodiment, new members within an Apop family are identified within the different cells or tissues of the same organism. The amino acid sequences of individual members are aligned and analyzed as described above. Amino acid sequences which are identical among those family members are used to design PCR primers. These PCR primers take the degeneracy of the genetic code into consideration and thus, may e.g., incorporate inosine at the third codon position. Similarly, PCR primers are designed based on the alignment and analysis of the respective nucleic acid sequences. Thus, PCR primers are designed to bind to conserved nucleotide sequences shared

by different members of the Apop family. In this embodiment, nucleotide sequences for all members of the Apop family, i.e., known and unknown members, sharing the respective nucleotide sequences to which the PCR primers bind, are generated by PCR. Subsequent subcloning and DNA analysis of the generated DNA fragments, as is well known in the art and e.g. described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989) and Ausubel et al., *Short Protocols in Molecular Biology* (John Wiley & Sons, Inc., 1995) identifies known and/or new members of the Apop family.

Once the Apop nucleic acid is identified, it can be cloned and, if necessary, its constituent parts recombined to form the entire Apop nucleic acid. Once isolated from its natural source, e.g., contained within a plasmid or other vector or excised therefrom as a linear nucleic acid segment, the recombinant Apop nucleic acid can be further-used as a probe to identify and isolate other Apop nucleic acids. It can also be used as a "precursor" nucleic acid to make modified or variant Apop nucleic acids and proteins.

Using the nucleic acids of the present invention which encode an Apop protein, a variety of expression vectors are made. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome. Generally, these expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the Apop protein. The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. The transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used to express the Apop protein, as will be appreciated

by those in the art; for example, transcriptional and translational regulatory nucleic acid sequences from *Bacillus* are preferably used to express the Apop protein in *Bacillus*. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells.

5 In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In a preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences.

10 Promoter sequences include constitutive and inducible promoter sequences. The promoters may be either naturally occurring promoters, hybrid or synthetic promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention.

15 In addition, the expression vector may comprise additional elements. For example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences which flank the expression construct. The integrating vector may be directed to a specific locus in the host  
20 cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors and appropriate selection and screening protocols are well known in the art and are described in e.g., Mansour et al., *Cell*, 51:503 (1988) and Murray, *Gene Transfer and Expression Protocols, Methods in Molecular Biology, Vol. 7* (Clifton: Humana Press, 1991).

25 In addition, in a preferred embodiment, the expression vector contains a selection gene to allow the selection of transformed host cells containing the expression vector, and particularly in the case of mammalian cells, ensures the stability of the vector, since cells which do not contain the vector will generally die. Selection genes are well known in the art and will vary with the host cell used. By "selection gene" herein is meant any gene which encodes a gene product that confers resistance to a selection agent. Suitable selection agents include, but are not limited to, neomycin (or its analog  
30 G418), blasticidin S, histidinol D, bleomycin, puromycin, hygromycin B, and other drugs.

In a preferred embodiment, the expression vector contains a RNA splicing sequence upstream or downstream of the gene to be expressed in order to increase the level of gene expression. See Barret et al., Nucleic Acids Res. 1991; Groos et al., Mol. Cell. Biol. 1987; and Budiman et al., Mol. Cell. Biol. 1988.

5 A preferred expression vector system is a retroviral vector system such as is generally described in Mann et al., Cell, 33:153-9 (1993); Pear et al., Proc. Natl. Acad. Sci. U.S.A., 90(18):8392-6 (1993); Kitamura et al., Proc. Natl. Acad. Sci. U.S.A., 92:9146-50 (1995); Kinsella et al., Human Gene Therapy, 7:1405-13; Hofmann et al., Proc. Natl. Acad. Sci. U.S.A., 93:5185-90; Choate et al., Human Gene Therapy, 7:2247 (1996); PCT/US97/01019 and PCT/US97/01048, and references cited therein,  
10 all of which are hereby expressly incorporated by reference.

The Apop proteins of the present invention are produced by culturing a host cell transformed with nucleic acid, preferably an expression vector, containing nucleic acid encoding an Apop protein, under the appropriate conditions to induce or cause expression of the Apop protein. The conditions appropriate for Apop protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation. For example, the use of constitutive promoters in the expression vector will require optimizing the growth and proliferation of the host cell, while the use of an inducible promoter requires the appropriate growth conditions for induction. In addition, in some embodiments, the timing of the harvest is important. For example, the baculoviral systems used in insect cell expression are lytic viruses, and thus harvest time selection can be crucial for product yield.

Appropriate host cells include yeast, bacteria, archaebacteria, fungi, and insect and animal cells, including mammalian cells. Of particular interest are *Drosophila melanogaster* cells, *Saccharomyces cerevisiae* and other yeasts, *E. coli*, *Bacillus subtilis*, SF9 cells, C129 cells, 293 cells, Neurospora, BHK, CHO, COS, and HeLa cells, fibroblasts, Schwannoma cell lines, immortalized mammalian myeloid  
25 and lymphoid cell lines, Jurkat cells, mast cells and other endocrine and exocrine cells, and neuronal cells. See the ATCC cell line catalog, hereby expressly incorporated by reference.

In a preferred embodiment, the Apop proteins are expressed in mammalian cells. Mammalian expression systems are also known in the art, and include retroviral systems. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream  
30 (3') transcription of a coding sequence for Apop3 protein into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, using a located 25-30 base pairs upstream of the transcription initiation site. The

TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter.

Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenylation signals include those derived from SV40.

The methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, is well known in the art, and will vary with the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, viral infection, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

In a preferred embodiment, Apop proteins are expressed in bacterial systems. Bacterial expression systems are well known in the art.

A suitable bacterial promoter is any nucleic acid sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of the coding sequence of Apop3 protein into mRNA. A bacterial promoter has a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region typically includes an RNA polymerase binding site and a transcription initiation site. Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose and maltose, and sequences derived from biosynthetic enzymes such as tryptophan. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters are also useful; for example, the *tac* promoter is a hybrid of the *trp* and *lac* promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription.



5 In addition to a functioning promoter sequence, an efficient ribosome binding site is desirable. In *E. coli*, the ribosome binding site is called the Shine-Delgarno (SD) sequence and includes an initiation codon and a sequence 3-9 nucleotides in length located 3 - 11 nucleotides upstream of the initiation codon.

10 The expression vector may also include a signal peptide sequence that provides for secretion of the Apop protein in bacteria. The signal sequence typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell, as is well known in the art. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria).

15 The bacterial expression vector may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed. Suitable selection genes include genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways.

These components are assembled into expression vectors. Expression vectors for bacteria are well known in the art, and include vectors for *Bacillus subtilis*, *E. coli*, *Streptococcus cremoris*, and *Streptococcus lividans*, among others.

20 The bacterial expression vectors are transformed into bacterial host cells using techniques well known in the art, such as calcium chloride treatment, electroporation, and others.

25 In one embodiment, Apop proteins are produced in insect cells. Expression vectors for the transformation of insect cells, and in particular, baculovirus-based expression vectors, are well known in the art and are described e.g., in O'Reilly et al., *Baculovirus Expression Vectors: A Laboratory Manual* (New York: Oxford University Press, 1994).

30 In a preferred embodiment, Apop protein is produced in yeast cells. Yeast expression systems are well known in the art, and include expression vectors for *Saccharomyces cerevisiae*, *Candida albicans* and *C. maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis* and *K. lactis*, *Pichia guillerimondii* and *P. pastoris*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*. Preferred promoter sequences for expression in yeast include the inducible GAL1,10 promoter, the promoters from alcohol

dehydrogenase, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase, hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, pyruvate kinase, and the acid phosphatase gene. Yeast selectable markers include ADE2, HIS4, LEU2, TRP1, and ALG7, which confers resistance to tunicamycin; the neomycin phosphotransferase gene, which confers resistance to G418; and the CUP1 gene, which allows yeast to grow in the presence of copper ions.

The Apop protein may also be made as a fusion protein, using techniques well known in the art. Thus, for example, for the creation of monoclonal antibodies, if the desired epitope is small, the Apop protein may be fused to a carrier protein to form an immunogen. Alternatively, the Apop protein may be made as a fusion protein to increase expression, or for other reasons. For example, when the Apop protein is an Apop peptide, the nucleic acid encoding the peptide may be linked to other nucleic acid for expression purposes.

In one embodiment, the Apop nucleic acids, proteins and antibodies of the invention are labeled. By "labeled" herein is meant that nucleic acids, proteins and antibodies of the invention have at least one element, isotope or chemical compound attached to enable the detection of nucleic acids, proteins and antibodies of the invention. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the compound at any position.

In a preferred embodiment, the Apop protein is purified or isolated after expression. Apop proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the Apop protein may be purified using a standard anti-Apop3 antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable purification techniques, see Scopes, R., Protein Purification, Springer-Verlag, NY (1982). The degree of purification necessary will vary depending on the use of the Apop3 protein. In some instances no purification will be necessary.

Once expressed and purified if necessary, the Apop proteins and nucleic acids are useful in a number of applications.

In a preferred embodiment, the Apop proteins are used to make Apop antibodies. Apop antibodies find use in a number of applications. For example, the Apop antibodies may be coupled to standard affinity

chromatography columns and used to purify Apop proteins. The antibodies may also be used as blocking polypeptides, as outlined above, since they will specifically bind to the Apop protein.

In a preferred embodiment, the Apop protein and/or Apop nucleic acid is used to generate antibodies, for example for immunotherapy. By "immunotherapy" is meant treatment of apoptosis with an antibody raised against Apop proteins. As used herein, immunotherapy can be passive or active. Passive immunotherapy, as defined herein, is the passive transfer of antibody to a recipient (patient). Active immunization is the induction of antibody and/or T-cell responses in a recipient (patient). Induction of an immune response can be the consequence of providing the recipient with an Apop antigen to which antibodies are raised. As appreciated by one of ordinary skill in the art, the Apop antigen may be provided by injecting an Apop polypeptide against which antibodies are desired to be raised into a recipient, or contacting the recipient with an Apop nucleic acid, capable of expressing the Apop antigen, under conditions for expression of the Apop antigen.

In a preferred embodiment, a therapeutic compound is conjugated to an antibody, preferably an Apop antibody. The therapeutic compound may be a cytotoxic agent. In this method, targeting the cytotoxic agent to apoptotic cells or tumor tissue or cells, results in a reduction in the number of afflicted cells, thereby reducing symptoms associated with apoptosis and cancer. Cytotoxic agents are numerous and varied and include, but are not limited to, cytotoxic drugs or toxins or active fragments of such toxins. Suitable toxins and their corresponding fragments include diphtheria A chain, exotoxin A chain, ricin A chain, abrin A chain, curcin, crotin, phenomycin, enomycin and the like. Cytotoxic agents also include radiochemicals made by conjugating radioisotopes to antibodies raised against Apop proteins, or binding of a radionuclide to a chelating agent that has been covalently attached to the antibody.

In a preferred embodiment, Apop genes are administered as DNA vaccines, either single genes or combinations of Apop genes. Naked DNA vaccines are generally known in the art; see Brower, *Nature Biotechnology* 16:1304-1305 (1998). Methods for the use of genes as DNA vaccines are well known to one of ordinary skill in the art, and include placing an Apop gene or portion of an Apop gene under the control of a promoter for expression in a patient. The Apop gene used for DNA vaccines can encode full-length Apop proteins, but more preferably encodes portions of the Apop proteins including peptides derived from the Apop protein. In a preferred embodiment a patient is immunized with a DNA vaccine comprising a plurality of nucleotide sequences derived from a Apop gene. Similarly, it is possible to immunize a patient with a plurality of Apop genes or portions thereof, as defined herein. Without being bound by theory, following expression of the polypeptide encoded by the DNA vaccine, cytotoxic T-cells, helper T-cells and antibodies are induced which recognize and destroy or eliminate cells expressing Apop proteins.

In a preferred embodiment, the DNA vaccines include a gene encoding an adjuvant molecule with the DNA vaccine. Such adjuvant molecules include cytokines that increase the immunogenic response to the Apop polypeptide encoded by the DNA vaccine. Additional or alternative adjuvants are known to those of ordinary skill in the art and find use in the invention.

5 In a preferred embodiment, the Apop proteins, nucleic acids, modified proteins and cells containing the native or modified Apop proteins are used in screening assays. Identification of the Apop proteins permits the design of drug screening assays for compounds that modulate Apop activity.

Screens are designed to first find candidate agents that can bind to Apop proteins, and then these agents are used in assays that evaluate the ability of the candidate agent to modulate Apop activity.

10 The terms "binding to Apop" and "modulating Apop activity" or grammatical equivalents thereof comprise binding to Apop1 proteins, Apop2 proteins, Apop3 proteins, and modulating Apop1 protein activity, Apop2 protein activity, and Apop3 protein activity. As will be appreciated by those in the art, there are a number of different assays which may be run; binding assays and activity assays.

15 In a preferred embodiment, the methods comprise combining an Apop protein and a candidate bioactive agent, and determining the binding of the candidate agent to the Apop protein. Preferred embodiments utilize the human Apop protein, although other mammalian Apop proteins may also be used, including rodents (mice, rats, hamsters, guinea pigs, etc.), farm animals (cows, sheep, pigs, horses, etc.) and primates. These latter embodiments may be preferred in the development of animal models of human disease. In some embodiments, as outlined herein, variant or derivative Apop proteins may be used, including deletion Apop proteins as outlined above. Furthermore, included within the definition of Apop proteins are portions or fragments of Apop proteins; that is, either the full-length protein may be used, or, as outlined above, portions or fragments thereof. In addition, the assays described herein may utilize either isolated Apop proteins or cells comprising the Apop proteins.

25 Generally, in a preferred embodiment of the methods herein, the Apop protein or the candidate agent, is non-diffusably bound to an insoluble support having isolated sample receiving areas (e.g. a microtiter plate, an array, etc.). The insoluble support may be made of any composition to which the Apop protein or the candidate agent can be bound, is readily separated from soluble material, and is otherwise compatible with the overall method of screening. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports include microtiter plates, arrays, membranes and beads. These are typically made of glass, plastic (e.g., polystyrene), polysaccharides, nylon or nitrocellulose, teflon™, etc. Microtiter plates and arrays are especially

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convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. The particular manner of binding the Apop protein or the candidate agent is not crucial so long as it is compatible with the reagents and overall methods of the invention, maintains the activity of the composition and is nondiffusable. Preferred methods of binding include the use of antibodies (which do not sterically block either the ligand binding site or activation sequence when the protein is bound to the support), direct binding to "sticky" or ionic supports, chemical crosslinking, the synthesis of the protein or agent on the surface, etc. Following binding of the Apop protein or candidate agent, excess unbound material is removed by washing. The sample receiving areas may then be blocked through incubation with bovine serum albumin (BSA), casein or other innocuous protein or other moiety.

In a preferred embodiment, the Apop protein is bound to the support, and a candidate bioactive agent is added to the assay. Alternatively, the candidate agent is bound to the support and the Apop protein is added. Novel binding agents include specific antibodies, non-natural binding agents identified in screens of chemical libraries, peptide analogs, etc. Of particular interest are screening assays for agents that have a low toxicity for human cells. Determination of the binding of an Apop protein and a candidate agent is done using a wide variety of assays, including, but not limited to labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.) and the like.

The term "candidate bioactive agent" or "exogeneous compound" as used herein includes any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, lipid, etc., or mixtures thereof, with the capability of directly or indirectly altering the bioactivity of Apop. Generally a plurality of assay mixtures is run in parallel with different candidate agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids,

purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

In a preferred embodiment, the candidate bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradations.

In a preferred embodiment, the candidate bioactive agents are naturally occurring proteins or fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eucaryotic proteins may be made for screening against Apop. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

In a preferred embodiment, the candidate bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below)

are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous agents.

5 In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or  
10 large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment a library of protein encoding nucleotide sequences is added to the host cell comprising the vector composition of the invention. The library of protein encoding nucleotide sequences may be obtained from genomic DNA, from cDNAs or from random nucleotides.

Particularly preferred in this embodiment are libraries encoding bacterial, fungal, viral, and mammalian proteins and peptides, with the latter being preferred, and human encoding proteins and peptides being especially preferred. As described above and as known in the art the protein and peptide encoding nucleotide sequences may be inserted into any vector suitable for expression in mammalian cells.

20 In a preferred embodiment, the candidate bioactive agents are nucleic acids. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage et al., Tetrahedron 49(10):1925  
25 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-  
30 methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference).

Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowski et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribonucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc.

As described above generally for proteins, nucleic acid candidate bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes may be used as is outlined above for proteins.

In a preferred embodiment, the candidate bioactive agents are obtained from combinatorial chemical libraries, a wide variety of which are available in the literature. By "combinatorial chemical library" herein is meant a collection of diverse chemical compounds generated in a defined or random manner, generally by chemical synthesis. Millions of chemical compounds can be synthesized through combinatorial mixing.

The determination of the binding of the candidate bioactive agent to the Apop protein may be done in a number of ways. In a preferred embodiment, the candidate bioactive agent is labeled, and binding determined directly. For example, this may be done by attaching all or a portion of the Apop protein to



a solid support, adding a labeled candidate agent (for example a candidate agent comprising a fluorescent label), washing off excess reagent, and determining whether the label is present on the solid support. Various blocking and washing steps may be utilized as is known in the art.

By "labeled" herein is meant that the candidate bioactive agent is either directly or indirectly labeled with a label which provides a detectable signal, e.g. a radioisotope (such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ ), a fluorescent or chemiluminescent compound (such as fluorescein isothiocyanate, rhodamine, or luciferin), an enzyme (such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase), antibodies, particles such as magnetic particles, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal.

In some embodiments, only one of the components is labeled. For example, the proteins (or proteinaceous candidate agents) may be labeled at tyrosine positions using  $^{125}\text{I}$ , or with fluorophores. Alternatively, more than one component may be labeled with different labels; using  $^{125}\text{I}$  for the proteins, for example, and a fluorophor for the candidate agents.

In a preferred embodiment, the binding of the candidate bioactive agent is determined through the use of competitive binding assays. In this embodiment, the competitor is a binding moiety known to bind to the target molecule (i.e. the Apop protein), such as an antibody, peptide, binding partner, ligand, etc.

In a preferred embodiment, the competitor for Apop3 is RIP (Stanger et al., *supra*). In another preferred embodiment, the competitor for Apop1 and Apop2 is XIAP (Deveraux et al., *Nature* 388:300-303 (1997)). Under certain circumstances, there may be competitive binding as between the bioactive agent and the binding moiety, with the binding moiety displacing the bioactive agent.

In one embodiment, the candidate bioactive agent is labeled. Either the candidate bioactive agent, or the competitor, or both, is added first to the protein for a time sufficient to allow binding, if present. Incubations may be performed at any temperature which facilitates optimal activity, typically between  $4^{\circ}\text{C}$  and  $40^{\circ}\text{C}$ . Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high through put screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

In a preferred embodiment, the competitor is added first, followed by the candidate bioactive agent. Displacement of the competitor is an indication that the candidate bioactive agent is binding to the Apop protein and thus is capable of binding to, and potentially modulating, the activity of the Apop protein. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the candidate bioactive agent is labeled, the presence of the label on the support indicates displacement.

In an alternative embodiment, the candidate bioactive agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate that the bioactive agent is bound to the Apop protein with a higher affinity. Thus, if the candidate bioactive agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate that the candidate agent is capable of binding to the Apop protein.

In a preferred embodiment, the methods comprise differential screening to identify bioactive agents that are capable of modulating the activity of the Apop proteins. In this embodiment, the methods comprise combining an Apop protein and a competitor in a first sample. A second sample comprises a candidate bioactive agent, an Apop protein and a competitor. Addition of the candidate bioactive agent is performed under conditions which allow the modulation of the Apop protein. The binding of the competitor is determined for both samples, and a change, or difference in binding between the two samples indicates the presence of an agent capable of binding to the Apop protein and potentially modulating its activity. That is, if the binding of the competitor is different in the second sample relative to the first sample, the agent is capable of binding to the Apop protein.

Another preferred embodiment utilizes differential screening to identify drug candidates that bind to the native Apop protein, but cannot bind to modified Apop proteins. The structure of the Apop protein may be modeled, and used in rational drug design to synthesize agents that interact with that site. Drug candidates that affect Apop bioactivity are also identified by screening drugs for the ability to either enhance or reduce the activity of the protein.

Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the protein. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

5 A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding.

10 Screening for agents that modulate the activity of Apop may also be done. In a preferred embodiment, methods for screening for a bioactive agent capable of modulating the activity of Apop comprise the steps of adding a candidate bioactive agent to a sample of Apop, as above, and determining an alteration in the biological activity of Apop. "Modulating the activity of Apop" includes an increase in activity, a decrease in activity, or a change in the type or kind of activity present. Thus, in this embodiment, the candidate agent should both bind to Apop (although this may not be necessary), and alter its biological or biochemical activity as defined herein. The methods include both in vitro screening methods, as are generally outlined above, and in vivo screening of cells for alterations in the presence, distribution, activity or amount of Apop.

15 Thus, in this embodiment, the methods comprise combining an Apop sample and a candidate bioactive agent, and evaluating the effect on apoptosis. By "Apop activity" or grammatical equivalents herein is meant one or more of the biological activities of Apop1, Apop2, and Apop3. Apop1's and Apop2's biological activity, e.g., comprises the ability to affect apoptosis. Apop3's biological activities, include, but are not limited to, its kinase activity, its ability to activate NF- $\kappa$ B, its ability to activate caspase-3, and its ability to affect apoptosis. Apop3 kinase activity may be assayed using known serine/threonine kinase assays; see Thome et al., J. Exp. Med 181:1997 (1995); del Peso et al., Science 278:687 (1997), and others. Similarly, death domain assays are known in the art.

20 In a preferred embodiment, the activity of the Apop protein is increased; in another preferred embodiment, the activity of the Apop protein is decreased. Thus, bioactive agents that are antagonists are preferred in some embodiments, and bioactive agents that are agonists may be preferred in other embodiments.

25 In a preferred embodiment, the invention provides methods for screening for bioactive agents capable of modulating the activity of an Apop protein. The methods comprise adding a candidate bioactive agent, as defined above, to a cell comprising Apop proteins. Preferred cell types include almost any cell as defined above for host cells. Preferred cells include, but are not limited to prokaryotic cells and eukaryotic cells, with mammalian cells and particularly human cells being preferred.

The cells contain a recombinant nucleic acid that encodes an Apop protein. In a preferred embodiment, a library of candidate agents is tested on a plurality of cells. By a "plurality of cells" herein is meant roughly from about  $10^3$  cells to  $10^8$  or  $10^9$ , with from  $10^6$  to  $10^8$  being preferred.

In some embodiments, the assays include exposing the cells to an apoptosis agent that will induce apoptosis in control cells, i.e. cells of the same type but that do not contain the exogenous nucleic acid encoding an Apop. Suitable apoptosis inducing agents are known in the art. Alternatively, the cells may be exposed to conditions that normally result in apoptosis, and changes in the normal apoptosis progression are determined. Alternatively, the cells into which the Apop nucleic acids are introduced, normally undergo apoptosis, and thus changes (for example, inhibition of apoptosis) are determined. Optionally, the cells normally do not undergo apoptosis, and the introduction of a candidate agent causes apoptosis.

Thus, the effect of the candidate agent on apoptosis is then evaluated.

Methods for the detection of apoptosis may be done as will be appreciated by those in the art. In one embodiment, indicators of apoptosis are used. Suitable apoptosis labels include, but are not limited to, DAPI. Accordingly, these agents can be used as an affinity ligand, and attached to a solid support such as a bead, a surface, etc. and used to pull out cells that are undergoing apoptosis. Similarly, these agents can be coupled to a fluorescent dye such as PerCP, and then used as the basis of a fluorescent-activated cell sorting (FACS) separation.

Sensitive assays that measure various biological and morphological hallmarks of the apoptotic process are known in the art. For example, a monoclonal antibody which can be used to detect cleavage of poly (ADP-ribose) polymerase, CCP32/Caspase-3 fluorescent and colorimetric assay kits, and a FLICE/caspase-8 fluorescent assay kit are commercially available (Clontech Laboratories, Inc.). Annexin V apoptosis assays, measuring the translocated phosphatidylserine (PS) are described, e.g., in Dachary-Prigent et al., Blood 81:2554-65 (1993); Thiagarajan and Tait, J. Biol. Chem. 265:17420-3 (1990); and Zhang et al., Biotechniques 23:525-31 (1997). Identification of apoptosis in situ via specific labeling of nuclear DNA fragmentation is described in Gavrieli et al., J. Cell Biol. 119:493-501 (1992).

In a preferred embodiment, bioactive agents identified by one of the methods outlined above, may be compounds showing pharmacological activity or therapeutical activity. Compounds with pharmacological activity are able to enhance or interfere with the activity of the Apop protein. The

compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host, as previously described. The agents may be administered in a variety of ways, orally, parenterally e.g., subcutaneously, intraperitoneally, intravascularly, etc. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of a therapeutically active compound in the formulation may vary from about 0.1-100 wt.%. 5

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents. 10

Without being bound by theory, it appears that the Apop proteins are important proteins in apoptosis. Accordingly, disorders based on mutant or variant Apop genes may be determined. In one embodiment, the invention provides methods for identifying cells containing variant Apop genes comprising determining all or part of the sequence of at least one endogenous Apop genes in a cell. As will be appreciated by those in the art, this may be done using any number of sequencing techniques. In a preferred embodiment, the invention provides methods of identifying the Apop genotype of an individual comprising determining all or part of the sequence of at least one Apop gene of the individual. This is generally done in at least one tissue of the individual, and may include the evaluation of a number of tissues or different samples of the same tissue. The method may include comparing the sequence of the sequenced Apop gene to a known Apop gene, i.e. a wild-type gene (e.g., those nucleic acid sequences depicted in Figures 1, 3, and 5) and determining if any differences exist. This can be done using any number of known homology programs, such as Bestfit, etc. In a preferred embodiment, the presence of a difference in the sequence between the Apop gene of the patient and the known Apop gene is indicative of a disease state or a propensity for a disease state, as outlined herein. 15 20 25

The present discovery relating to the role of Apop in apoptosis thus provides methods for inducing or preventing apoptosis in cells. In a preferred embodiment, the Apop proteins, and particularly Apop fragments, are useful in the study or treatment of conditions which are mediated by apoptosis, i.e. to diagnose, treat or prevent apoptosis-mediated disorders. Thus, "apoptosis mediated disorders" or "disease state" include conditions involving both insufficient apoptosis, including cancer, autoimmune 30

disorders and sustained viral infections, and excessive apoptosis, including inappropriate cell loss and degenerative disorders.

In one embodiment, methods of modulating apoptosis in cells or organisms are provided. In one embodiment, the methods comprise administering to a cell an anti-Apop antibody that reduces or eliminates the biological activity of the endogenous Apop protein. Alternatively, the methods comprise administering to a cell or organism a recombinant nucleic acid encoding an Apop protein. As will be appreciated by those in the art, this may be accomplished in any number of ways. In a preferred embodiment, the activity of Apop is increased by increasing the amount of Apop in the cell, for example by overexpressing the endogenous Apop or by administering a gene encoding Apop, using known gene-therapy techniques, for example. In a preferred embodiment, the gene therapy techniques include the incorporation of the exogenous gene using enhanced homologous recombination (EHR), for example as described in PCT/US93/03868, hereby incorporated by reference in its entirety.

In one embodiment, the invention provides methods for diagnosing an Apop mediated disorder in an individual. The methods comprise measuring the activity and expression of Apop in a tissue from an individual or patient, which may include a measurement of the amount or specific activity of Apop. This activity is quantified and compared to the activity of Apop from either an unaffected second individual or from an unaffected tissue from the first individual. When these activities are different, the first individual may be at risk for an Apop mediated disorder. In this way, for example, monitoring of various disease conditions may be done, by monitoring the levels of Apop. Similarly, Apop levels may correlate to the diseases and conditions enumerated above.

In one aspect, the expression levels of Apop genes are determined in different patient samples or cells for which either diagnosis or prognosis information is desired. Gene expression monitoring is done on genes encoding Apop proteins. In one aspect, the expression levels of Apop genes are determined for different cellular states, such as normal cells and cells undergoing apoptosis. By comparing Apop gene expression levels in cells in different states, information including both up- and down-regulation of Apop genes is obtained, which can be used in a number of ways. For example, the evaluation of a particular treatment regime may be evaluated: does a chemotherapeutic drug act to improve the long-term prognosis in a particular patient. Similarly, diagnosis may be done or confirmed by comparing patient samples. Furthermore, these gene expression levels allow screening of drug candidates with an eye to mimicking or altering a particular expression level. This may be done by making biochips comprising sets of important Apop genes, such as those of the present invention, which can then be used in these screens. These methods can also be done on the protein basis; that is, protein

expression levels of the Apop proteins can be evaluated for diagnostic purposes or to screen candidate agents. In addition, the Apop nucleic acid sequences can be administered for gene therapy purposes, including the administration of antisense nucleic acids, or the Apop proteins administered as therapeutic drugs.

5 Apop sequences bound to biochips include both nucleic acid and amino acid sequences as defined above. In a preferred embodiment, nucleic acid probes to Apop nucleic acids (both the nucleic acid sequences having the sequences outlined in Figures 1, 3, and 5 and/or the complements thereof) are made. The nucleic acid probes attached to the biochip are designed to be substantially  
10 complementary to the Apop nucleic acids, i.e. the target sequence (either the target sequence of the sample or to other probe sequences, for example in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur  
15 under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions, particularly high stringency conditions, as outlined herein.

20 A "nucleic acid probe" is generally single stranded but can be partially single and partially double stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. In general, the nucleic acid probes range from about 8 to about 100 bases long, with from about 10 to about 80 bases being preferred, and from about 30 to about 50 bases being particularly preferred. In some embodiments, much longer nucleic acids can be used, up to hundreds of bases (e.g., whole genes).

25 As will be appreciated by those in the art, nucleic acids can be attached or immobilized to a solid support in a wide variety of ways. By "immobilized" and grammatical equivalents herein is meant the association or binding between the nucleic acid probe and the solid support is sufficient to be stable under the conditions of binding, washing, analysis, and removal as outlined below. The binding can be covalent or non-covalent. By "non-covalent binding" and grammatical equivalents herein is meant one  
30 or more of either electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment of a molecule, such as, streptavidin to the support and the non-covalent binding of the biotinylated probe to the streptavidin. By "covalent binding" and grammatical equivalents herein is meant that the two moieties, the solid support and the probe, are attached by at

least one bond, including sigma bonds, pi bonds and coordination bonds. Covalent bonds can be formed directly between the probe and the solid support or can be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the probe or both molecules. Immobilization may also involve a combination of covalent and non-covalent interactions.

5 In general, the probes are attached to the biochip in a wide variety of ways, as will be appreciated by those in the art. As described herein, the nucleic acids can either be synthesized first, with subsequent attachment to the biochip, or can be directly synthesized on the biochip.

10 The biochip comprises a suitable solid substrate. By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of the nucleic acid probes and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates are very large, and include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, TeflonJ, etc.), polysaccharides, 15 nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, etc. In general, the substrates allow optical detection and do not appreciably show fluorescence.

20 In a preferred embodiment, the surface of the biochip and the probe may be derivatized with chemical functional groups for subsequent attachment of the two. Thus, for example, the biochip is derivatized with a chemical functional group including, but not limited to, amino groups, carboxy groups, oxo groups and thiol groups, with amino groups being particularly preferred. Using these functional groups, the probes can be attached using functional groups on the probes. For example, nucleic acids containing amino groups can be attached to surfaces comprising amino groups, for example using linkers as are known in the art; for example, homo-or hetero-bifunctional linkers as are well known 25 (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). In addition, in some cases, additional linkers, such as alkyl groups (including substituted and heteroalkyl groups) may be used.

30 In this embodiment, oligonucleotides, corresponding to the nucleic acid probe, are synthesized as is known in the art, and then attached to the surface of the solid support. As will be appreciated by those skilled in the art, either the 5' or 3' terminus may be attached to the solid support, or attachment may be via an internal nucleoside.



In an additional embodiment, the immobilization to the solid support may be very strong, yet non-covalent. For example, biotinylated oligonucleotides can be made, which bind to surfaces covalently coated with streptavidin, resulting in attachment.

Alternatively, the oligonucleotides may be synthesized on the surface, as is known in the art. For example, photoactivation techniques utilizing photopolymerization compounds and techniques are used. In a preferred embodiment, the nucleic acids can be synthesized in situ, using well known photolithographic techniques, such as those described in WO 95/25116; WO 95/35505; U.S. Patent Nos. 5,700,637 and 5,445,934; and references cited within, all of which are expressly incorporated by reference; these methods of attachment form the basis of the Affimetrix GeneChip™ technology.

"Differential expression," or grammatical equivalents as used herein, refers to both qualitative as well as quantitative differences in the genes' temporal and/or cellular expression patterns within and among the cells. Thus, a differentially expressed gene can qualitatively have its expression altered, including an activation or inactivation, in, for example, normal versus apoptotic cell. That is, genes may be turned on or turned off in a particular state, relative to another state. As is apparent to the skilled artisan, any comparison of two or more states can be made. Such a qualitatively regulated gene will exhibit an expression pattern within a state or cell type which is detectable by standard techniques in one such state or cell type, but is not detectable in both. Alternatively, the determination is quantitative in that expression is increased or decreased; that is, the expression of the gene is either upregulated, resulting in an increased amount of transcript, or downregulated, resulting in a decreased amount of transcript. The degree to which expression differs need only be large enough to quantify via standard characterization techniques as outlined below, such as by use of Affymetrix GeneChip™ expression arrays, Lockhart, Nature Biotechnology 14:1675-1680 (1996), hereby expressly incorporated by reference. Other techniques include, but are not limited to, quantitative reverse transcriptase PCR, Northern analysis and RNase protection.

As will be appreciated by those in the art, this may be done by evaluation at either the gene transcript, or the protein level; that is, the amount of gene expression may be monitored using nucleic acid probes to the DNA or RNA equivalent of the gene transcript, and the quantification of gene expression levels, or, alternatively, the final gene product itself (protein) can be monitored, for example through the use of antibodies to the Apop protein and standard immunoassays (ELISAs, etc.) or other techniques, including mass spectroscopy assays, 2D gel electrophoresis assays, etc.

In another method detection of the mRNA is performed in situ. In this method permeabilized cells or tissue samples are contacted with a detectably labeled nucleic acid probe for sufficient time to allow

the probe to hybridize with the target mRNA. Following washing to remove the non-specifically bound probe, the label is detected. For example a digoxigenin labeled riboprobe (RNA probe) that is complementary to the mRNA encoding an Apop protein is detected by binding the digoxigenin with an anti-digoxigenin secondary antibody and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

In another preferred method, expression of Apop proteins is performed using in situ imaging techniques employing antibodies to Apop proteins. In this method cells are contacted with from one to many antibodies to the Apop protein(s). Following washing to remove non-specific antibody binding, the presence of the antibody or antibodies is detected. In one embodiment the antibody is detected by incubating with a secondary antibody that contains a detectable label. In another method the primary antibody to the Apop protein(s) contains a detectable label. In another preferred embodiment each one of multiple primary antibodies contains a distinct and detectable label. This method finds particular use in simultaneous screening for a plurality of Apop proteins. The label may be detected in a fluorometer which has the ability to detect and distinguish emissions of different wavelengths. In addition, a fluorescence activated cell sorter (FACS) can be used in this method. As will be appreciated by one of ordinary skill in the art, numerous other histological imaging techniques are useful in the invention and the antibodies can be used in ELISA, immunoblotting (Western blotting), immunoprecipitation, BIACORE technology, and the like.

In one embodiment, a therapeutically effective dose of an Apop is administered to a patient. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for Apop degradation, systemic versus localized delivery, and rate of new protease synthesis, as well as the age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals, and organisms. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

The administration of the Apop proteins of the present invention can be done in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intranasally, transdermally,

intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly. In some instances, for example, in the treatment of wounds and inflammation, the Apop may be directly applied as a solution or spray.

5 The pharmaceutical compositions of the present invention comprise an Apop protein in a form suitable for administration to a patient. In the preferred embodiment, the pharmaceutical compositions are in a water soluble form, such as being present as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, 10 sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. "Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, 20 tripropylamine, and ethanolamine.

The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

25 In another preferred embodiment, antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of Apop genes *in vivo*. The term "antisense" herein means that the nucleic acid sequence of the antisense RNA or DNA comprises the reverse complement sequence of the mRNA to which it can bind. For example, if the mRNA, whose expression (i.e., translation into a protein) is to be blocked, comprises the nucleic acid sequence 5'-GGAAUUGGAGC-3', then the 30 antisense RNA comprises the nucleic acid sequence of 5'-GCUCCAAUUCC-3' and the antisense DNA comprises the nucleic acid sequence of 5'-GCTCCAATTCC-3'.

5 An Apop antisense molecule as defined herein is a nucleic acid molecule which exhibits at least 75%, more preferably at least 80%, even more preferably at least 90%, and most preferably more than at least 95% reverse-complement identity to its target sequence. In one embodiment herein, an Apop antisense molecule exhibits 100% reverse-complement identity to its target sequence. As known in the art, the antisense nucleic acid can comprise nucleotide homologues, such as inosine, etc.

10 An Apop antisense molecule as defined herein is a nucleic acid molecule which inhibits expression or translation of an Apop nucleic acid by at least 30%, preferably by at least 40%, more preferably by at least 50%, even more preferably by at least 70%, and most preferably by at least 90%. In one embodiment herein, an Apop antisense molecule inhibits expression or translation of mRNA encoding Apop 100%.

15 Generally, the Apop antisense molecule is at least about 10 nucleotides in length, more preferably at least 12, and most preferably at least 15 nucleotides in length. The skilled artisan understands that the length can extend from 10 nucleotides or more to any length which does not inhibit binding to the Apop nucleic acid. In a preferred embodiment herein, the length is about 100 nucleotides long, more preferably about 50 nucleotides, more preferably about 25 nucleotides, and most preferably about 12 to 25 nucleotides in length.

20 It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane [Zamecnik et al., Proc. Natl. Acad. Sci. U.S.A. 83:4143-4146 (1986)]. The oligonucleotides can be modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups. There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection [Dzau et al., Trends in Biotechnology 11:205-210 (1993)]. In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target

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intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., J. Biol. Chem. 262:4429-4432 (1987); and Wagner et al., Proc. Natl. Acad. Sci. U.S.A. 87:3410-3414 (1990). For review of gene marking and gene therapy protocols see Anderson et al., Science 256:808-813 (1992).

5 In a preferred embodiment, the Apop proteins of the present inventions are used as "bait proteins" in a yeast two-hybrid screening or in a mammalian two-hybrid screening (Luo et al., Biotechniques 22:350-352 (1997) and US application SN 09/050,863, both of which are expressly incorporated as references in their entirety) to isolate novel proteins interacting with the Apop proteins.

10 The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

#### EXAMPLE 1

##### Isolation and sequence analysis of Apop3

15 Several known TNF $\alpha$  signaling proteins have been shown to bind RIP [Hsu et al., Immunity 4:387-396 (1996)] and complexes formed by the interactions between RIP kinase, TRADD, FADD, and RAIDD recruit other proteins to TNFR1 or Fas receptors to initiate signaling [Stanger et al., Cell 81:513-523 (1995); Hsu et al., Immunity 4:387-396 (1996); Tartaglia et al., Cell 74:845-853 (1993); Darnay et al., J. Leukoc. Biol. 61:559-566 (1997); Ashkenazi and Dixit, Science 281:1305-1308 (1998)]. We established a large-scale yeast two hybrid screening system designed to isolate rare mRNAs and verify novel protein-protein interactions involved in the TNF signaling pathway. Using RIP as bait, we screened 20 96 million independent yeast transformants with a combined HeLa/Lymphocyte cDNA library in a single transformation round. As expected, many of the known RIP binding proteins, including TRAF1, TRAF2, 25 TNFR1, RIP, TRADD, and FADD, were cloned; several novel genes were isolated as well. One of the novel cDNAs had high sequence homology with RIP. The full-length cDNA (Figure 5) contains a 518 amino acid open reading frame, encoding a protein designated Apop3. Analysis of the amino acid sequence of this novel protein (Figure 6) revealed a kinase domain (aa 21-287) which had 47% homology to the kinase domain of RIP and 42% homology to that of RIP2/Rick/CARDIAK. However, 30 outside the kinase domain, the C-terminal region of Apop3 had no homology to death domains [Tartaglia et al., Cell 74:845-853 (1993)], death effector domains [Siegel et al., J. Cell. Biol. 141:1243-1253 (1998)], or the CARD region [Hofmann et al., Trends Biochem. Sci. 22:155-156 (1997)]. An approximately 2.1

Kb transcript was detected with a cDNA probe specific to Apop3 in both normal human tissue and human tumor RNA blots (data not shown).

## EXAMPLE 2

### Co-immunoprecipitation with Apop3 and other members of TNF $\alpha$ signaling complex

5 We confirmed the association of Apop3 and other members of TNF $\alpha$  signaling complex in mammalian cells by co-immunoprecipitation. To further analyze which region of Apop3 was important for interactions with RIP, a series of deletion mutants were engineered (Figure 7). In yeast, the intermediate region (aa 82–436) of Apop3 was required for the interaction between RIP and Apop3 (Figure 8). Co-immunoprecipitation analysis was performed to verify the interaction of RIP with Apop3 by co-transfection of HA-tagged RIP with the Flag-tagged full length and truncated mutants of Apop3 in Phoenix-A (293 T) cells. In this example, cell lysates were collected from Phoenix-A cells 24 hours after the co-transfection of HA-RIP (5  $\mu$ g) with a Flag-control protein or with one of the Flag-Apop3 proteins, schematically depicted in Figure 7 (5  $\mu$ g). Co-precipitated Flag-Apop3 proteins were detected by immunoblotting with anti-Flag polyclonal Ab. The full length Apop3 co-immunoprecipitated with RIP, as well as Apop3(82-518) and Apop3(K50D) mutants (data not shown). Interestingly, the Apop3(1-436) mutant has dramatically reduced binding affinity for RIP. No association was detected between RIP and Apop3(1-251), Apop3(1-196), or Apop3(287-518). This suggests that the interaction between Apop3 and RIP is mediated by a portion of the kinase domain with contribution from the carboxyl-domain of Apop3.

## EXAMPLE 3

### Apop3 binds to endogenous RIP

20 To test whether Apop3 was able to bind to endogenous RIP, Flag-Apop3 was transfected into Phoenix-A (293 T) cells in the presence and absence of TNF $\alpha$  stimulation. In this example, 293 T cells were transfected with Flag-control protein, and Flag-Apop3 (10  $\mu$ g). After 18 hours incubation, cells were treated with or without 20 ng/ml huTNF $\alpha$  for 6 hours. The cell extracts were harvested 24 hours after transfection. The immunoprecipitated proteins were immunoblotted with goat anti-RIP polyclonal Ab. Ectopically expressed Apop3 was able to bind to endogenous RIP kinase (data not shown). TNF $\alpha$  treatment did not significantly affect this binding (data not shown).

## EXAMPLE 4

### Endogenous Apop3 binds to TRAF2 and TNFR1

In addition, endogenous TNFR1 was co-immunoprecipitated with ectopically expressed Apop3 and endogenous Apop3 was co-immunoprecipitated with ectopically expressed TRAF2 (data not shown). Interaction between TRAF2 and Apop3 was also verified by the yeast two-hybrid system (data not shown). This suggests that Apop3 is part of the TNFR1 signaling complex. In this example, Flag-tagged TRAF2 expression vector was transfected into cells. Association with endogenous Apop3 was detected by anti-Apop3 Ab. Further, Flag-tagged Apop3 was transfected into cells and association with endogenous TNFR1 was detected by anti-TNFR1 Ab (data not shown)

#### EXAMPLE 5

##### Demonstration of APO3 kinase activity

Apop3 contains consensus Ser/Thr kinase motifs. The lysine 50 of the Apop3 kinase domain, homologous to the conserved lysine in RIP and critical for enzymatic activity and ATP-binding [McCarthy et al., J. Biol. Chem. 273:16968-16975 (1998); Inohara et al., J. Bio. Chem. 273: 12296-12300 (1998)], was substituted with aspartic acid. An *in vitro* kinase assay demonstrated that Apop3 was a kinase and was autophosphorylated (data not shown). However, the Apop3(K50D) mutant lost the kinase activity (data not shown), suggesting that this lysine is required for autophosphorylation. In this example, Phoenix-A cells were transfected with 10 µg of a Flag-vector, Flag-Apop3 and Flag-Apop3(K50D), respectively. The lysates were immunoblotted with anti-Flag mAb to adjust protein amount for IP.

#### EXAMPLE 6

##### Induction of cell death by Apop3

To test whether Apop3 was able to induce cell death, Phoenix-A cells were co-transfected with a eukaryotic expression vector encoding Apop3 and a pGDB control for transfection efficiency [Xu et al., Nucl. Acids Res. 26:2034-2035 (1998)]. The GFP-positive Apop3-transfected cells displayed morphological characteristics of apoptosis, including detachment from the plate substrate and resultant "rounding up" and a condensed/fragmented nucleus [Cryns et al., Cell 82:349-352 (1995)] (data not shown). The C-terminal deletion mutant, Apop3(1-436), failed to induce apoptosis. Apop3-induced apoptosis was also observed in Hela cells (data not shown). Figure 9 shows approximately 35% cell death in the Apop3 transfected Phoenix-A cells, compared with only 2% apoptosis in cells transfected with control vector. Similar to RIP [Hsu et al., Immunity 4:387-396 (1996)] and RIP2/Rick/CARDIAK kinase deletions [McCarthy et al., J. Biol. Chem. 273:16968-16975 (1998)], the deletion of the Apop3 kinase domain had no effect on its pro-apoptotic activity (data not shown), which suggested the kinase domain was not required for Apop3-mediated cell death.

#### EXAMPLE 7

### Activation of cellular caspases by Apop3

TNF $\alpha$  and Fas ligand induced apoptosis is controlled by caspase activation [Martin and Green, Cell 82:349-352 (1995)]. Rick/RIP2/CARDIAK kinase has also been reported to induce apoptosis through enhancement of caspase activity [Inohara et al., J. Bio. Chem. 273: 12296-12300 (1998)]. To test whether Apop3 activates cellular caspases, pYCI-Apop3 (data not shown) was co-transfected into Phoenix-A cells with pGDB. In this example,  $2 \times 10^6$  Phoenix-A cells were co-transfected with expression vectors encoding Apop3 and Apop3 mutants, such as depicted in Figure 7 (10  $\mu$ g) with pGDB (3 $\mu$ g). Lysates were analyzed by Western blot probed with anti-GFP monoclonal antibodies. Caspase cleavage released the monomeric GFP as indicated by the arrow. pGDB expresses a previously described BFP-GFP hybrid protein linked by a DEVD containing peptide ('GFP'-GSGSGSDEV DGGSGSGS-'BFP', wherein the caspase cleavage site is in between V and D) used as a reporter of intracellular caspase activity [Xu et al., Nucl. Acids Res. 26:2034-2035 (1998)]. When caspases 2, 3, or 7 are activated, they specifically cleave appropriately accessible substrates at the DEVD peptide [Cryns et al., Cell 82:349-352 (1995)] and release GFP/BFP monomers. Western blot analysis using anti-GFP monoclonal antibody indicated that ectopic expression of Apop3 activated caspases to cleave the DEVD linker peptide, releasing GFP/BFP monomer (data not shown). A broad-spectrum inhibitor of caspases, zVAD-fmk [Zhu et al., FEBS Lett. 374:303-308 (1995)], inhibited the caspase activity triggered by Apop3 expression (data not shown). This result confirmed that the cleavage of the chimeric protein was due to caspase activation (data not shown). Apop3-induced apoptosis could also be inhibited by CrmA (data not shown). N-terminal deletion mutants Apop3(82-518), Apop3(287-518) and Apop3(K50D) were still able to activate the caspase activity (data not shown). Deletion of the C-terminal region of Apop3, Apop3(1-251) and Apop3(1-436), completely abrogated caspase activation (data not shown). These results indicate again that Apop3's kinase activity is not required for activation of caspases and cell death. Since the C-terminal domain (aa 437-518) of Apop3 is not required for the binding of Apop3 to RIP, this region may be involved in binding to other proteins involved in novel signaling pathway(s) leading to apoptosis. It is not known whether the C-terminal region of Apop3 is able to bind directly to known death adaptor or death protease. Additional two-hybrid screening using Apop3 as bait is being carried out to find signaling proteins downstream of Apop3.

### EXAMPLE 8

#### Induction of activation of NF $\kappa$ B by Apop3

We also tested whether Apop3 could induce NF $\kappa$ B activation. Phoenix-A cells were harvested for a luciferase reporter assay 24 hours after co-transfection with an NF $\kappa$ B-dependent luciferase reporter construct and an Apop3 expression vector (pYCI-Apop3; data not shown). Ectopic expression of Apop3 alone induced NF $\kappa$ B activation approximately 7 fold (Figure 10 and data not shown). The NF $\kappa$ B activation



could be inhibited by co-transfection of a I $\kappa$ B dominant negative mutant (data not shown). The C-terminal region was essential for NF $\kappa$ B activation, as Apop3(1-251) and Apop3(1-436) were not able to activate NF $\kappa$ B (Figure 10). Interestingly, Apop3(K50D), Apop3(82-518), and Apop3(287-518) induced higher luciferase activity than full length Apop3 (Figure 10). This suggests that the kinase domain of Apop3 is not required for the NF $\kappa$ B activation. In contrast, Apop3 had no noticeable effect on AP-1 activation pathways (data not shown).

#### EXAMPLE 9

##### Overexpression of Apop3 inhibits TNF $\alpha$ -induced caspase activation but not TNF $\alpha$ -induced NF $\kappa$ B activation

Although Apop3 associates with RIP and TRAF2 and activates apoptosis and NF $\kappa$ B, its function in the TNF $\alpha$  signaling pathway needs to be dissected. In this example, Phoenix-A (293 T) cells were co-transfected with expression vectors encoding Apop3 and Apop3 mutants, such as depicted in Figure 7 (3 $\mu$ g) with pGDB (0.3  $\mu$ g) and RIP (0.1  $\mu$ g). After 24 hours of transfection, cell lysates were harvested and analyzed by Western blot analysis with anti-GFP monoclonal antibodies. In these experiments, Apop3(1-436) inhibited TNF $\alpha$ -induced caspase activation (data not shown) but not TNF $\alpha$ -induced NF $\kappa$ B activation (data not shown), suggesting that Apop3 is involved in the TNF $\alpha$ -mediated apoptosis pathway. These results imply that without the C-terminal domain (aa 437-518), Apop3 either competes with or fails to recruit other proteins of the TNF signaling of a complex required for the activation of caspases. In separate experiments, Apop3(1-436) was able to down-regulate RIP-mediated apoptosis activation (data not shown). Co-transfection of full length of Apop3 (3  $\mu$ g) with RIP (0.1  $\mu$ g) resulted in greater caspase activity than with RIP alone (data not shown). However, Apop3(1-436) significantly reduced RIP-induced caspase activity (data not shown). On the other hand, although Apop3 alone was able to activate NF $\kappa$ B, co-transfection of RIP (0.3  $\mu$ g) and full length Apop3 (3  $\mu$ g) in Phoenix cells did not show any synergistic effect on NF $\kappa$ B activation (data not shown). When Apop3(1-436) was co-transfected with RIP, it only slightly reduced RIP-mediated NF $\kappa$ B activation (data not shown). Another Apop3 mutant (aa 287-518) was still able to activate caspase and NF $\kappa$ B, even though it did not bind to RIP (data not shown).

In summary, we demonstrated that Apop3 is a novel kinase associated with the TNF $\alpha$  signaling complex. Apop3 binds to RIP and TRAF2 in both yeast and mammalian cells and co-immunoprecipitates with TNFR1. Although RIP is an essential player in TNF $\alpha$  induced NF $\kappa$ B activation [Kelliher et al., Immunity 8:297-303 (1998)] and Apop3 was cloned by binding to RIP, the function of Apop3 may not rely solely on its binding to RIP. A dominant negative form of Apop3 is able to inhibit TNF $\alpha$ -induced caspase activation without affecting TNF $\alpha$ -induced NF $\kappa$ B activation, suggesting that Apop3 may be involved in TNF $\alpha$ -induced apoptosis. It is possible that Apop3 and RIP are therefore involved in two different

areas of TNF $\alpha$  signaling; apoptosis and NF $\kappa$ B activation, respectively. The precise role of Apop3 in TNF $\alpha$  signaling may be answered by future knock-out experiments.

#### EXAMPLE 10

##### The yeast two-hybrid screening method: Plasmids, cDNA libraries, yeast strains, growth media and retrieval of plasmids

Unknown proteins interacting with a known "Bait" protein can be identified using the yeast two-hybrid screening method. In this method, a known "Bait" protein (such as XIAP or RIP) is encoded on a "bait vector" and a protein (the "Test" protein) which may bind to the "Bait" protein is usually encoded on a "test" vector or "test" plasmid. The method comprises the following steps: (1) transforming yeast with a "bait" vector and a "test" vector (usually a library); (2) detecting an interaction between the bait protein and a test protein (usually by expression of a reporter gene such as lacZ); (3) growing the identified yeast colony; (4) isolating the "test" vector; (5) transforming the "test" vector into E.coli; and (6) using standard techniques to further characterize the "test" vector and the encoded "test" protein.

Plasmids and cDNA Libraries: The pAS2 and pACT2 plasmid series were originally constructed by Elledge and co-workers [Durfee et al., Genes Dev. 4:555-69 (1993)]. The bait vector used in our screening is either pAS2 or pAS2K. pAS2K was constructed by replacing the Amp<sup>R</sup> gene in pAS2 with a Kan<sup>R</sup> gene. cDNA libraries used in our screening were made, e.g., either from brain tissues or T and B lymphocytes.

The yeast two-hybrid system reporter strain Y190 (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4D, gal80D, cyhr2, LYS2::GAL1UAS-HIS3TATA-HIS3, URA3::GAL1UAS-GAL1TATA-lacZ) was used in all screenings.

All yeast culture mediums, including, e.g., YPD, YPD Agar, DOB, DOBA, CSM-TRP, CSM-LEU, CSM-HIS, CSM-URA, CSM-LYS, CSM-LEU-TRP, CSM-LEU-HIS, and CSM-LEU-TRP-HIS, are available from Bio101, Inc. 3AT (3-amino-1,2,4-triazol) is available from Sigma (Cat# A-8056, St. Louis, MO, USA).

There are several methods to retrieve plasmids from yeast, ranging from lyticase lysis to glass beads. The glass beads method is listed below:

1. Inoculate 3 ml of selection medium (e.g., SD-L for cDNA library plasmid pACT) with a yeast colony.
2. Incubate in a 30°C shaker or rotator overnight or until confluent.
3. Spin down yeast in a bench-top centrifuge at 3000 rpm at room temperature.

-50-

4. Remove medium and re-suspend pellet in 200  $\mu$ l lysis buffer. Transfer to an eppendorf tube.
5. Add 200  $\mu$ l volume glass beads.
6. Add 200  $\mu$ l phenol/chloroform/isoamyl alcohol (25:24:1).
7. Vortex at the highest speed for 3 minutes.
- 5 8. Spin in micro-centrifuge at 14000 rpm for 10 minutes.
9. Transfer top water layer to another eppendorf tube, add 20  $\mu$ l 3M NaAc and 500  $\mu$ l ethanol.
10. Put the eppendorf tube into a dry ice bath for 15 minutes or until frozen.
11. Spin in a micro-centrifuge 14000 rpm for 10 minutes.
12. Remove supernatant and dry pellet.
- 10 13. Wash pellet by 100  $\mu$ l of 80% ethanol, and dry the pellet in air.
14. Re-suspend pellet in 30  $\mu$ l H<sub>2</sub>O and use 1 $\mu$ l for electroporation to transform *E. coli*.

Electroporation method is by far the most efficient method to transform plasmids from yeast miniprep into *E. coli*. Bait and cDNA (test) plasmids may carry different antibiotic selection markers to facilitate separation in *E. coli*. For example, our bait plasmid carries a Kan<sup>R</sup> gene and the cDNA (test) plasmid carries an Amp<sup>R</sup> gene.

ART 34 AMDT.

51

We claim:

1. A recombinant nucleic acid encoding an Apop3 protein that comprises an amino acid sequence at least 85% identical to the amino acid sequence depicted in Figure 6.
2. A recombinant nucleic acid according to claim 1 comprising the nucleotide sequence depicted in Figure 5 or its complement.
3. A recombinant nucleic acid according to claim 1 or 2 wherein said nucleic acid hybridizes under high stringency conditions to the nucleotide sequence depicted in Figure 5 or its complement.
4. A recombinant nucleic acid according to any of claims 1, 2, or 3 wherein said nucleic acid comprises a nucleotide sequence at least 85% identical to the nucleotide sequence depicted in Figure 5.
5. A recombinant nucleic acid according to any of claims 1-4 wherein said Apop3 protein comprises the amino acid sequence depicted in Figure 6.
6. A recombinant nucleic acid according to any of claims 1-5 wherein said Apop3 protein is a human Apop3 protein.
7. A recombinant nucleic acid comprising nucleotides 1-822 depicted in Figure 5, or its complement.
8. A recombinant nucleic acid according to any of claims 1-7 operably linked to control sequences recognized by a host cell transformed with the nucleic acid.
9. An expression vector comprising the nucleic acid of any of claims 1-8.
10. A host cell comprising the nucleic acid of any of claims 1-8.

11. A host cell comprising the expression vector of claim 9.
12. A recombinant Apop3 protein comprising an amino acid sequence at least 85% identical to the amino acid sequence depicted in Figure 6.
13. An Apop3 protein according to claim 12 comprising the amino acid sequence depicted in Figure 6.
14. An Apop3 protein according to claim 12 or 13 wherein said Apop3 protein is encoded by a nucleic acid comprising the nucleotide sequence depicted in Figure 5.
15. An Apop3 protein according to any of claims 12, 13, or 14 wherein said Apop3 protein is encoded by a nucleic acid which nucleic acid comprises a nucleotide sequence at least 85% identical to the nucleotide sequence depicted in Figure 5.
16. An Apop3 protein according to any of claims 12-15 wherein said Apop3 protein is encoded by a nucleic acid which nucleic acid will hybridize under high stringency conditions of the nucleotide sequence depicted in Figure 5 or its complement.
17. An Apop3 protein according to any of claims 12-16 wherein said Apop3 protein is a human Apop3 protein.
18. A recombinant Apop3 protein comprising the amino acid sequence 1-274 depicted in Figure 6.
19. A process for producing an Apop3 protein according to any of claims 12-18 comprising culturing the host cell of claim 10 or 11 under conditions suitable for expression of said Apop3 protein.
20. A process according to claim 19, further comprising recovering said Apop3 protein.
21. A monoclonal antibody which specifically binds to an Apop3 protein according to any

of claims 12-18.

22. A monoclonal antibody according to claim 21 that reduces or eliminates the biological function of said Apop3 protein.

23. A method for screening for a bioactive agent capable of binding to an Apop3 protein according to any of claims 12-17, said method comprising:

- a) combining said Apop3 protein and a candidate bioactive agent; and
- b) determining the binding of said candidate bioactive agent to said Apop3 protein.

24. A method for screening for a bioactive agent capable of interfering with the binding of an Apop3 protein. according to any of claims 12-17, and RIP, said method comprising:

- a) combining said Apop3 protein, a candidate bioactive agent and a RIP protein; and
- b) determining the binding of said Apop3 protein and said RIP protein.

25. A method for screening for a bioactive agent capable of modulating the activity of an Apop3 protein according to any of claims 12-17, said method comprising the steps of:

- a) adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding an Apop3 protein; and
- b) determining the effect of the candidate bioactive agent on apoptosis.

26. A method according to claim 25 wherein a library of candidate bioactive agents is added to a plurality of cells comprising a recombinant nucleic acid encoding said Apop3 protein.

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T06050.16429260

1 CACAGACAGGCTCTTAAGTGTCTTATATCACTCACTTGGGAAAGTGTCTCAAGCTGTTCTA  
61 CAAATCCATGCAAAGGCCGTTTAAAAATAGCAGCGAAGGTCCTGGACTCGGTCTCGTCCA  
121 GCACAGCCCCTTGGCTCTCTCTCTGGGCTCTGGCCGCTGGCCCCCGGGACCCACACGA  
181 GGTCAATGGCGTGTCTCGGGCAGGGGGGCGGGGATCCCATAGACACCTCAGCTCCTTAAGA  
241 GTTCTCCGCCTGGGCCAGGACGAGCATGGGGGTCCCCACTGATGCCCCGAGACAGTGCCCC  
301 TGTGTGTGTGAGCCCTCGACCCACATAACAGAGAGGTGTCTGATGCCCTCTGTCCTCTC  
361 CAGGTGGATCTAGGATCCGGCTTCCAACATGTGGCAGCTCTGGGCCTCCCTCTGCTGCCT  
421 GCTGGTGTGGCCAATGCCCGGAGCAGGCCCTCTTTCCATCCCGTGTGCGATGAGCTGGT  
481 CAACTATGTCAACAAACGGAATACCACGTGGCAGGCCGGGCACAACTTCTACAACGTGGA  
541 CATGAGCTACTTGAAGAGGCTATGTGGTACCTTCCTGGGTGGGCCCAAGCCACCCAGAG  
601 AGTTATGTTTACCGAGGACCTGAAGCTGCCTGCAAGCTTCGATGCACGGGAACAATGGCC  
661 ACAGTGTCCCACCATCAAAGAGATCAGAGACCAGGGCTCCTGTGGCTCCTGCTGGGTAAG  
721 GCCCTGCTGGCTGGTGGGGAAGCGCTGGAGAGAAAGTGGGAGCAACACTGGAGAGTCTTG  
781 GGGGATTCGGGGTGGGGACAACTCTGACAAGGCAAGTTATAGAACTTTCTGAGTCCCAG  
781 TTTCCATCAGTACAAAAATCACAATCCCTCTGGCCATGAATGATGGCGAGGATTAGGTGG  
841 AGTGGCGGGCAGAGCATCCAGCAGATTGCAAGTCCACGTGTACAGGTGGCGAAGCAGCTC  
901 CCTTTCCCTGACATGCTGGCCCGTCCGCAAATACCAGGAGCTCTCACTGCTACTCTGCTT  
961 CAAGAAAGCATCCCTTTAGTGTGAGTGAGCTGTCTTAATTTTGTCAATTAATTGTGGTAA  
1021 AATACACGTAACAGAAATGTAATAATCTTAGCAATCTTCTTTTGTCTTTCTTTTCTTTT  
1081 TTTTTTTTTTTTTTTT

FIGURE 1

1 MWQLWASLCCLLVLANARSRPSFHPVSDLVNYVNKRNTTWQAGHNFYNVDMSYLKRLCG  
61 TFLGGPKPPQRVMFTEDLKLPASFDAREQWPQCPTIKEIRDQGSCGSCWVRPCWLVGKRW  
121 RESGSNTGESWGIRGGDNSDKASYRNFLSPSFHQYKNHNPSGHE

## FIGURE 2

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1 TCCAAGTCCTGGATGAGCTCGGAAGACACGTGCTGCTGCGGAAGGACTGTGGCCCAGTGG  
61 ACACCAAGGTTCCAGGTGCTGGGGAGCCCCAAGTCAGCCTTCACTCAGGGCTCAGCCATGA  
121 TTTCTTCTCTGTCCCAAAACCATCCAGACAACAGAAACGAGACTTTCAGCTCAGTCATTT  
181 CCCTGTTGAATGAGGATCCCCTGTCCCAGGACTTGCCCTGTGAAGATGGCTTCAATCTTCA  
241 AGAACTTTGTCAATTACCTATAACCGGACATATGAGTCAAAGGAAGAAGCCCGGTGGCGCC  
301 TGTCGGTCTTTGTCAATAACATGGTGCGAGCACAGAAGATCCAGGCCCTGGACCGTGGCA  
361 CAGCTCAGTATGGAGTCACCAAGTTCAGTGATCTCACAGAGGAGGAGTCCGCACTATCT  
421 ACCTGAATACTCTCCTGAGGAAAGAGCCTGGCAACAAGATGAAGCAAGCCAAGTCTGTGG  
481 GTGACCTCGCCCCACCTGAATGGGACTGGAGGAGTAAGGGGGCTGTCACAAAAGTCAAAG  
541 ACCAGGGCATGTGTGGCTCCTGCTGGGCCTTCTCAGTCACAGGCAATGTGGAGGGCCAGT  
601 GGTTTCTCAACCAGGGGACCCTGCTCTCCCTCTCTGAACAGGAGCTCTTGAGCTGTGACA  
661 AGATGGACAAGGCCTGCATGGGCGGCTTGCCCTCCAATGCCTACTCGGCCATAAAGAATT  
721 TGGGAGGGCTGGAGACAGAGGATGACTACAGCTACCAGGGTCACATGCAGTCCTGCAACT  
781 TCTCAGCAGAGAAGGCCAAGGTCTACATCAATGACTCCGTGGAGCTGAGCCAGAACGAGC  
841 AGAAGCTGGCAGCCTGGCTGGCCAAGAGAGGCCCAATCTCCGTGGCCATCAATGCCTTTG  
901 GCATGCAGTTTTACCGCCACGGGATCTCCCGCCCTCTCCGGCCCCCTCTGCAGCCCTTGGC  
961 TCATTGACCATGCGGTGTTGCTTGTGGGCTACGGCAACCGCTCTGACGTTCCCTTTTGGG  
1021 CCATCAAGAACAGCTGGGGCACTGACTGGGGTGAGAAGGGTTACTACTACTTGCATCGCG  
1081 GGTCCGGGGCCTGTGGCGTGAACACCATGGCCAGCTCGGCGGTGGTGGACTGAAGAGGGG  
1141 CCCCCAGCTCGGGACCTGGTGCTGATCAGAGTGGCTGCTGCCCCAGCCTGACATGTGTCC  
1201 AGGCCCCCTCCCCGGGAGGTACAGCTGGCAGAGGGAAAGGCACTGGGTACCTCAGGGTGAG  
1261 CAGAGGGCACTGGGCTGGGGCACAGCCCCTGCTT

## FIGURE 3

1 MISSLSQNHDPNRNETFSSVISLLNEDPLSQDLPVKMASIFKNFVITYNRTYESKEEARW  
61 RLSVFVNNMVRQAQKIQALDRGTAQYGVTKFSDLTEEEFRTIYLNNTLLRKEPGNKMKAQS  
121 VGD LAPPEWDWRSKGAVTKVKDQGMCGSCWAFSVTGNVEGWFLNQGTLLSLSEQELLDC  
181 DKMDKACMGGLPSNAYSAIKNLGGLETEDDYSYQGHMQSCNFSAEKAKVYINDSVELSON  
241 EQKLAAWLAKRGPI SVAINAFGMQFYRHGISRPLRPLCSPWLIDHAVLLVGYGNRSDVPF  
301 WAIKNSWGTDWGEKGYYYLHRGSGACGVNTMASSAVVD

**FIGURE 4**

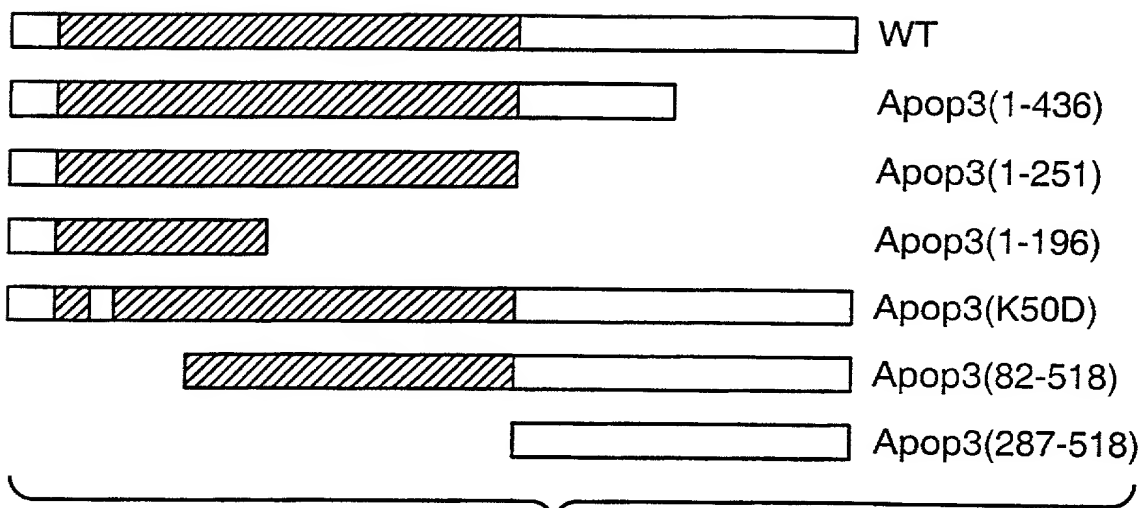
1 atgtcgtgcg tcaagttatg gcccagcggg gcccccgccc ccttggtgtc catcgaggaa  
 61 ctggagaacc aggagctcgt cggcaaagac ggggttcggca cagtgttccg ggcgcaacat  
 121 aggaagtggg gctacgatgt ggcgggtcaag atcgtaaact cgaaggcgat atccagggag  
 181 gtcaaggcca tggcaagtct ggataacgaa ttcgtgttgc gcctagaagg ggttatcgag  
 241 aaggtgaact gggaccaaga tcccaagccg gctctggtga ctaaattcat ggagaacggc  
 301 tccctgtcgg ggctgctgca gtcccagtcg cctcgccctt ggccgctcct ttgccgcctg  
 361 ctgaaagaag tgggtgcttg gatgttttac ctgcacgacc agaaccgggt gctcctgcac  
 421 cgggacctca agccatccaa cgtcctgccg gacccagagc tgcacgtcaa gctggcagat  
 481 tttggcctgt ccacatttca gggaggctca cagttaggga cagggtccgg ggagccaggg  
 541 ggcaccctgg gctacttggc ccagaactg tttgttaacg taaaccggaa ggcctccaca  
 601 gccagtgcag tctacagctt cgggaccta atgtgggcag tgcttgctgg aagagaagtt  
 661 gagttgcaa ccgaaccatc actcgtgtac gaagcagtgt gcaacaggca gaaccggcct  
 721 tcattggctg agctgcccc aagcgggcct gagactcccg gcttagaagg actgaaggag  
 781 ctaatgcagc tctgctggag cagttagccc aaggacagac cctccttcca ggaatgccta  
 841 caaaaaactg atgaagtctt ccagatgggtg gagaacaata tgaatgctgc tgtctccacg  
 901 gtaaaggatt tcctgtctca gctcaagagc agcaatagga gattttctat cccagagtca  
 961 ggccaaggag ggacagaaat ggatggcttt aggagaacca tagaaaacca gcactctcgt  
 1021 aatgatgtca tggtttctga gtggctaaac aaactgaatc tagaggagcc tcccagctct  
 1081 gttcctaaaa aatgcccagc ccttaccaag aggagcaggg cacaagagga gcaggttcca  
 1141 caagcctgga cagcaggcac atcttcagat tcgatggccc aacctcccca gactccagag  
 1201 acctcaactt tcagaaacca gatgcccgcc cctacctcaa ctggaacacc aagtccctgga  
 1261 ccccgagggg atcagggggc tgagagacaa ggcattgaact ggtcctgcag gaccccgag  
 1321 ccaaatccag taacaggggc accgctcgtt aacatataca actgctctgg ggtgcaagtt  
 1381 ggagacaaca actacttgac tatgcaacag acaactgcct tgcccacatg gggcttgga  
 1441 ccttcgggca aggggagggg cttgcagcac cccaccag taggttcgca agaaggcct  
 1501 aaagatcctg aagcctggag caggccacag gttggtata atcatagcg gaaataa

FIGURE 5

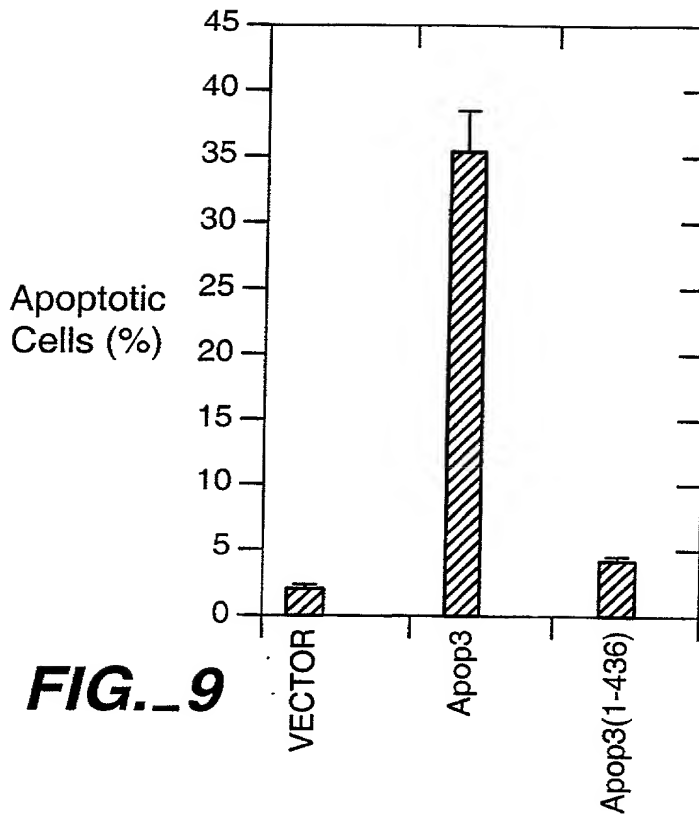
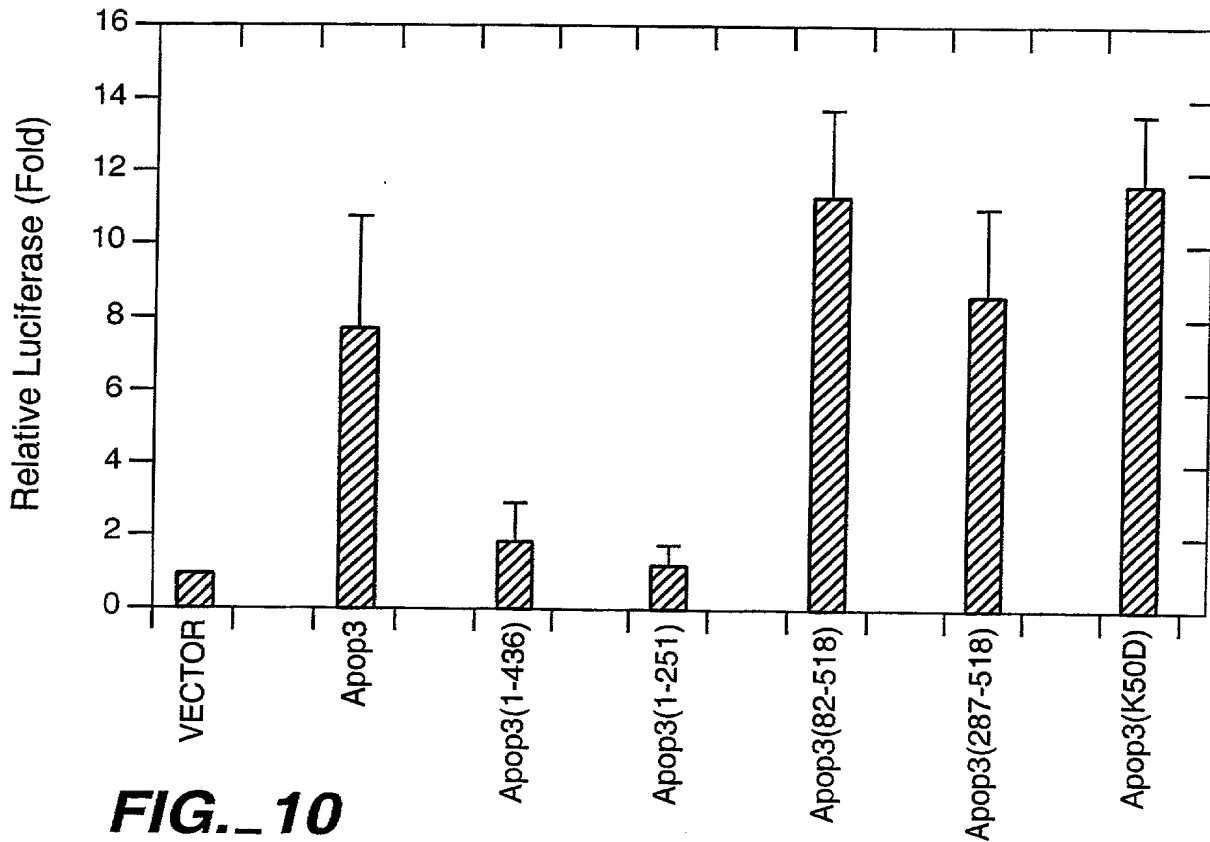
1 MSCVKLWPSGAPAPLVSIEELENQELVGKDGFGTVFRAQHRKWGYDVAVKIVNSKAISRE  
61 VKAMASLDNEFVLRLEGVIEKVNWDQDPKPALVTKFMENGSLSGLLQSQCPRPWPLLCLRL  
121 LKEVVLGMFYLHDQNPVLLHRDLKPSNVLDPDELHVKLADFGGLSTFQGGSSQSGTGSSEPG  
181 GTLGYLAPEL FVNVRKASTASDVYSFGILMWAVLAGREVELPTEPSLVYEAVCNRQNRP  
241 SLAELPQAGPETPGLEGLKELMQLCWSSEPKDRPSFQECLPKTDEVFQMVENNMNAAVST  
301 VKDFLSQLKSSNRRFSIPESGQGGTEMDGFRRTIENQHSRNDVMVSEWLNKLNLLEPPSS  
361 VPKKCPSLTKRSRAQEEQVPQAWTAGTSSDSMAQPPQTPTSTFRNQMPSPSTSTGTPSPG  
421 PRGNQGAERQGMNWSCRTPEPNPVTGRPLVNIYNCSGVQVGDNNYLTMQQTALPTWGLA  
481 PSGKGRGLQHPPVGSQEGPKDPEAWSRPQGWNHSGKZ

[illegible]

## FIGURE 6



		Binding To RIP
<b>FIG._8</b>	Apop3	++++
	Apop3(1-436)	+
	Apop3(1-251)	-
	Apop3(1-196)	-
	Apop3(K50D)	++++
	Apop3(82-518)	+++
	Apop3(287-518)	-

**FIG. 9****FIG. 10**

**POWER OF ATTORNEY BY ASSIGNEE**

The undersigned assignee of the entire interest in application for letters patent entitled:  
**NOVEL APOPTOSIS PROTEINS**  
and having the named inventors:  
Ying Luo, Betty Huang, Mary Shen and PeiWen Yu

and described in

- ☒ the specification filed herewith  
☐ application serial no. \_\_\_\_\_ filed \_\_\_\_\_  
☐ patent no. \_\_\_\_\_ issued \_\_\_\_\_

hereby appoints the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith; said appointment to be to the exclusion of the inventor(s) and his (their) attorney(s) in accordance with the provisions of 37 C.F.R. 1.32:

Harold C. Hohbach	<del>17,757</del>	James A. Sheridan	<del>25,435</del>	Edward N. Bachand	<del>37,085</del>
Aldo J. Test	<del>18,048</del>	Robert B. Chickering	<del>24,286</del>	R. Michael Ananian	<del>35,050</del>
Donald N. MacIntosh	<del>20,316</del>	Richard F. Trecartin	<del>31,801</del>	Robin M. Silva	<del>38,304</del>
Edward S. Wright	<del>24,903</del>	Steven F. Caserza	<del>29,780</del>	Maria S. Swiatek	<del>37,244</del>
David J. Brezner	<del>24,774</del>	Michael A. Kaufman	<del>32,988</del>	Julian Caplan	<del>14,785</del>
Richard E. Backus	<del>22,701</del>	Janet E. Muller	<del>35,294</del>	Brian T. Clarke	<del>45,552</del>

(18)

provided that if any one of said attorneys ceases being affiliated with the law firm of Flehr Hohbach Test Albritton & Herbert LLP as partner, employee or of counsel, such attorney's appointment as attorney and all powers derived therefrom shall terminate on the date such attorney ceases being so affiliated.

In accordance with 37 CFR 3.73 the assignee hereby certifies that the evidentiary documents with respect to its ownership have been reviewed and that, to the best of assignee's knowledge and belief, title is in the assignee seeking to take this action.

Direct all telephone calls to Robin M. Silva, Esq. at (415) 781-1989.

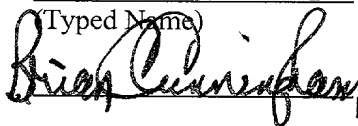
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By: Brian C. Cunningham  
(Typed Name)

Signature:



Title: Sr. Vice President, COO & CFO

Address: 240 East Grand Avenue, S. San Francisco, CA 94080

Date 02/02/01

File No. A-68285/RMS/DHR

09762491 050901

## DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled NOVEL APOPTOSIS PROTEINS the specification of which

(check one) ☐ is attached hereto.

☐ was filed on \_\_\_\_\_ as  
Application Serial No. \_\_\_\_\_  
and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>PCT/US99/17776</u>	<u>August 6, 1999</u>	<u>Pending</u>
(Application Serial No.)	(Filing Date)	(Status)
(patented, pending, abandoned)		



<u>60/099,486</u>	<u>September 8, 1998</u>	<u>Pending</u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)
<u>60/095,590</u>	<u>August 6, 1998</u>	<u>Pending</u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)
<u>60/095,587</u>	<u>August 6, 1998</u>	<u>Pending</u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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